The role of PI3Kγ and PI3K adaptor protein Bam32 in neutrophil recruitment and microvascular hyperpermeability

A Thesis

Submitted to the College of

Graduate and Postdoctoral Studies

In Partial Fulfillment of the Requirements

For the Degree of Doctor of Philosophy

In the Department of Anatomy, Physiology, and Pharmacology

University of Saskatchewan

Canada

By

Li HAO

PERMISSION TO USE

In presenting this thesis in partial fulfillment of the requirements for a Postgraduate degree from the University of Saskatchewan, I agree that the libraries of this University may make it freely available for inspection. I further agree that permission for copying of this thesis in any manner, in whole or in part, for scholarly purposes may be granted by the professor or professors who supervised my thesis work or, in their absence, by the Head of the Department or the Dean of College of Medicine in which my thesis work was done. It is understood that any copying or publication or use of this thesis or parts thereof for financial gain shall not be allowed without my written permission. It is also understood that due recognition shall be given to me and to the University of Saskatchewan in any scholarly use which may be made of any material in my thesis.

Requests for permission to copy or to make other use of the material in this thesis in whole or part should be addressed to:

Head of the Department of Anatomy, Physiology, and Pharmacology
College of Medicine, University of Saskatchewan
Saskatoon, Saskatchewan S7N 5E5
Canada
OR
Dean
College of Graduate and Postdoctoral Studies
University of Saskatchewan
116 Thorvaldson Building, 110 Science Place
Saskatoon, Saskatchewan, S7N 5C9
Canada

ABSTRACT

B cell adaptor molecule of 32 kDa (Bam32), also known as dual adapter for phosphotyrosine and 3-phosphoinositides 1 (DAPP1), is an adaptor protein downstream of phosphoinositide-3-kinase (PI3K) signaling pathway. Although the roles of Bam32 have been revealed in some subsets of leukocytes during the past two decades, its role in neutrophils remains completely unknown. In my study, I respectively explored the roles of Bam32 and its upstream PI3K subunits in intermediary chemoattractant CXCL2-induced and end-target chemoattractant Trp-Lys-Tyr-Met-Val-D-Met-NH2 (WKYMVm)-induced neutrophil recruitment, neutrophil reactive oxygen species (ROS) production, and microvascular hyperpermeability in mice.

Firstly, I revealed that PI3Kγ, an upstream regulator of Bam32, plays a critical role in neutrophil-driven microvascular leakage induced by WKYMVm. Using intravital microscopy in mice, I found a PI3Kγ-specific mechanism in WKYMVm-induced but not CXCL2-induced microvascular hyperpermeability. The increased microvascular permeability triggered by WKYMVm was not entirely due to neutrophil adhesion and emigration in mouse cremasteric microvasculature. The PI3Kγ-specific hyperpermeability was neutrophil-mediated as this was reduced after depletion of neutrophils in mouse circulation. Chimeric mice with PI3Kγ-deficient neutrophils but wild-type (WT) endothelium also showed reduced microvascular hyperpermeability. Furthermore, I found that the catalytic function of PI3Kγ was required for reactive oxygen species (ROS) generation in neutrophils stimulated with WKYMVm. Pharmacological scavenging PI3Kγ-dependent ROS in the tissue eliminated the discrepancy in hyperpermeability between different PI3K transgenic mice and alleviated WKYMVm-induced microvascular leakage in all mouse strains tested.

Secondly, I uncovered a role for Bam32 in regulating CXCL2-induced neutrophil recruitment through small GTPase Rap1. Using intravital microscopy in mouse cremaster muscle, I found that transmigrated neutrophil number, neutrophil chemotaxis velocity and total neutrophil chemotaxis distance were increased in Bam32$^{-/-}$ mice when compared with WT mice. In CXCL2-induced mouse peritonitis, the total emigrated neutrophils were increased in Bam32$^{-/-}$ mice at 2 h but not 4 h. CXCL2-induced chemotaxis distance and migration velocity of isolated Bam32$^{-/-}$ neutrophils

in vitro were increased. I examined the activation of small GTPases Rac1 and Rap1, the expression levels of phospho-Akt2 and total Akt2 and their crosstalk with Bam32 in neutrophils. The deficiency of Bam32 resulted in the elevation of Rac1-GTP binding and suppression of Rap1-GTP binding. Pharmacological inhibition of Rac1 by NSC23766 markedly reduced activation of Rap1 in WT neutrophils and reduced Bam32$^{-/-}$ neutrophil chemotaxis, whereas inhibition of Rap1 by GGTI298 increased WT neutrophil chemotaxis. In addition, deficiency of Bam32, as well as inhibition of Rap1 activation, increased the levels of CXCL2-induced Akt1/2 phosphorylation at Thr308/309 in neutrophils. Inhibition of Akt by SH-5 attenuated CXCL2-induced adhesion and emigration in Bam32$^{-/-}$ mice.

Thirdly, I unveiled a role for Bam32 in modulating WKYMVm-induced formation of microvascular hyperpermeability through the production of neutrophil ROS. I observed significantly reduced WKYMVm-induced microvascular hyperpermeability accompanied by markedly decreased neutrophil emigration only in Bam32$^{-/-}$ mice. The Bam32-specific decrease in WKYMVm-induced hyperpermeability was neutrophil-dependent as this was verified in bone marrow transplanted Bam32 chimeric mice. I also discovered that Bam32 was critically required for WKYMVm-induced intracellular and extracellular production of ROS in neutrophils. Pharmacological scavenging of ROS eliminated the differences in WKYMVm-induced hyperpermeability between Bam32$^{-/-}$ and WT mice. In addition, the deficiency of Bam32 reduced WKYMVm-induced ERK1/2 but not p38 or JNK phosphorylation in neutrophils. Inhibition of ERK1/2 signaling cascade suppressed WKYMVm-induced ROS generation in WT neutrophils and microvascular hyperpermeability in WT mice.

Taken together, my study shows both PI3Kγ and PI3K downstream adaptor Bam32 regulate WKYMVm-induced microvascular hyperpermeability through modulating ROS production in neutrophils. Moreover, Bam32 suppresses CXCL2-induced neutrophil recruitment through Rap1 activation. My study provides insights regarding PI3K signaling pathway for regulating neutrophil recruitment, neutrophil ROS production, and microvascular barrier function in acute inflammation.

ACKNOWLEDGMENTS

After a journey of six years rafting in the ocean of science, many greatest thanks should be sent, at this moment before the finishing line, to those people who watched me, supported me, and cheered for me all the way.

First of all, I would like to give the sincerest thanks to my supervisor Dr. Lixin Liu. Had you not put that recruiting poster on the university website six years ago, I would never have this opportunity to join your lab and start my study as a PhD student. I do appreciate your time spent with me in your office discussing the progress of my research, your suggestions and criticisms that perfected my academic writing skills, your generous financial support that helped me feed my family, and your trust that encouraged me to dig into the research area I am interested in. Your diligence and preciseness will always be the merits I should learn no matter in which field I will be devoted in the future.

I would like to express my gratitude to Dr. Aaron Marshall in the University of Manitoba for the technical help in some experiments, the scientific inputs in shaping my manuscripts, and the valuable suggestions in responding to the reviewers.

I would like to extend my thanks to my committee members Dr. Stanley Bardal for letting me involve in the tutorial session of DENT392, Dr. Kaushik Desai for sharing all the equipment in your lab and providing me very important guidance, Dr. Venkat Gopalakrishnan for generously helping me when I was struggling with my PCOL850 course, Dr. John Gordon for raising sharp questions that reminded me to stay hungry and keep humble, and Dr. Changiz Taghibiglou for always providing valuable suggestions that inspired me to think much more after the seminars and committee meetings.

I would like to thank Cindy Thomson and Dawn Giesbrecht for giving me the chance to work in BMSC240 course as a teaching assistant for two years. I am so happy to be able to work in your team and do appreciate your efforts to create such a comfortable working environment. This job not only enabled me to learn how the top undergraduates absorb new knowledge, but more

importantly gave me the feeling of coming down to earth and helping people. Also, I would extend my thanks to Glenn Hamonic and Akanksha Baharani for your frank and valuable suggestions on my future career.

I would like to thank my colleagues Bo Jiang for helping me with the troubles inside and outside the lab, and my former colleagues Yang Su, Md. Mokarram Hossain, and Musstafa Smeir for sharing me your precious experience in experiments. I would like to thank the lab manager Heather Neufeld for providing great assistance in every new experiment I was trying to set up, and all my friends on the second floor for making the life colorful in every single day.

I would also like to thank my friends Ang Chen in the College of Engineering, Yiyan Li in the College of Arts and Science, Jinlai Chen in the College of Nursing, Feifei Han on the road, and other friends who have left Saskatoon. We came to this city in the beautiful summer of 2014 and met in the course GSR981. I do cherish the days we spent together playing board games in my home and the trips we made during the past years. I am happy to see we are all launching our new lives and do hope all you guys have bright futures.

I would express very special thanks to my parents Xiaohong Hao and Min Zhang for your unconditional support. I know you always trust me and try to give me the best you have. Your son will never let you down.

Lastly, for my wife Xia Zhong and my kids Charlotte Hao and Aaron Hao, what I have is not merely a “thank-you” but all my love. I love you more than you may know. I love you because you are you.

DEDICATION

To my parents

Xiaohong HAO and Min ZHANG

For the unconditional support

To my wife

Xia ZHONG

For the company through every storm in our journey

To my daughter and my son

Charlotte HAO and Aaron HAO

For the happiness we have together

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

Bam32	B cell adapter molecule of 32 kDa
BCR	B cell receptor
CXCL	CXC chemokine ligand
CXCR	CXC chemokine receptor
DAPP1	dual adaptor for phosphotyrosine and 3-phosphoinositides 1
DC	dendritic cell
DCF-DA	2',7'– dichlorofluorescin diacetate
DM	diabetes mellitus
DPI	diphenyleneiodonium
ERK	extracellular regulated protein kinase
eNOS	endothelial nitric oxide synthase
FGF2	fibroblast growth factor 2
FI	fluorescence intensity
FITC	fluorescein isothiocyanate
fMLP	N-formylmethionyl-leucyl-phenylalanine
FPR	formyl peptide receptor
GAPs	GTPase activating proteins
GEFs	guanine nucleotide exchange factors
GM-CSF	granulocyte macrophage colony-stimulating factor
GROb	growth-related oncogene-b
GTPase	guanosine triphosphatase
HIF	hypoxia inducible factor
ICAM-1	intercellular adhesion molecule-1
JAM-C	junction adhesion molecule C precursor
JNK	Jun N-terminal kinase
KI	knock-in
KO	knock-out
LFA-1	lymphocyte function-associated antigen-1
LPS	lipopolysaccharide
LSP1	leukocyte-specific protein 1

LTB_4	leukotriene B_4
Mac-1	macrophage-1 antigen
MAPK	mitogen-activated protein kinase
MAPKAPK	MAPK activated protein kinase
MLCK	myosin light-chain kinase
MRSA	methicillin-resistant *Staphylococcus aureus*
NADPH	nicotinamide adenine dinucleotide phosphate
NETs	neutrophil extracellular traps
NO	nitric oxide
NOX	NADPH oxidase
PAF	platelet-activating factor
PAMPs	pathogen-associated molecular patterns
PCR	polymerase chain reaction
PH domain	pleckstrin homology domain
PI3K	phosphoinositide-3-kinase
PI(3,4,5)P3	phosphatidylinositol 3,4,5-triphosphate
PKC	protein kinase C
PLC	phospholipase C
PTP	protein tyrosine phosphate
Rac	Ras-related C3 botulinum toxin substrate
Rap	Ras-related protein
ROCK	Rho-associated coiled-coil kinase
ROS	reactive oxygen species
RNS	reactive nitrogen species
RSK	ribosomal S6 kinases
rAC	reverse abluminal crawling
rIM	reverse interstitial migration
rLC	reverse luminal crawling
rTEM	reverse transendothelial migration
S1P	sphingosine 1-phosphate
Ser	serine
SH2	src homology 2

SHIP	src homology 2-containing inositol 5'-phosphatase
SOD	superoxide dismutase
STAT5	signal transducer and activator of transcription 5
TNFα	tumor necrosis factor-α
TCR	T cell receptor
Thr	threonine
TLR	Toll-like receptor
VCAM-1	vascular cell adhesion molecule 1
VEGF	vascular endothelial growth factor
WKYMVm	Trp-Lys-Tyr-Met-Val-D-Met-NH_2
WT	wild-type

CHAPTER ONE

INTRODUCTION

1.1 Neutrophils in inflammation—the first-line defensive cells in innate immunity

1.1.1 Neutrophils

Neutrophils, a group of polymorphonuclear leukocytes, are derived from hematopoietic stem cells, matured in and released from the bone marrow and circulate physiologically in the bloodstream [1,2]. They are recruited to inflammatory sites and perform their characteristic functions during acute inflammation. Neutrophils, differentiated from myeloid precursors, take up to 50%–70% of total leukocytes in human blood circulation. However, in mouse circulation, neutrophils only account for 10%–25% of total leukocytes albeit still being predominant [3,4]. The average half-life of neutrophils is extremely short, generally believed as 1.5 h for mouse neutrophils and 8 h for human neutrophils in physiological conditions [5]. Nevertheless, a recent study reported that the circulatory lifespan of a mouse neutrophil might be up to 12.5 h, and that of a human neutrophil might be up to 5 days [6]. In pathological conditions such as inflammation, the longevity of both mouse and human neutrophils extends dramatically to allow neutrophils to carry out their biological functions in the circulation [1,7].

Neutrophils have three types of granules: primary (azurophilic) granules, secondary (specific) granules and tertiary (gelatinase) granules. The primary granules contain many bactericidal and cytotoxic enzymes that help in both intracellular and extracellular digestion. The specific granules contain enzymes performing similar biological functions as the enzymes in the primary granules, but also contain NADPH oxidase, which is a critical enzyme for neutrophil oxidative burst. The tertiary granules contain enzymes such as gelatinase B that facilitate the degradation of the basement membrane of tissues [8]. The enzymes in neutrophils that perform synergetic functions

are stored separately in different types of granules, because some of them will start to react immediately with each other if they meet under certain conditions [9].

Compared to the well-defined cell structures in neutrophils, the number of neutrophil subsets is still debatable. Aside from the normal neutrophils in the circulation of wild-type mice, a previous study reported that a new type of pro-inflammatory neutrophils was found in methicillin-resistant *Staphylococcus aureus* (MRSA)-resistant mice and a new type of anti-inflammatory neutrophils in MRSA-susceptible mice [10]. These three types of mouse neutrophils contained identical cytokines and chemokines but were distinct on the nuclear morphology, surface receptors, and adhesion molecules. This report challenges the prevailing view that neutrophils do not have subsets, but it is still too early to declare the existence of different types of neutrophils because it was proven that neutrophils can change the expression of chemokine receptors and adhesion molecules in some situations [11].

Regardless of the subsets, neutrophils clear pathogens through various killing mechanisms, both extracellularly and intracellularly. They can either release antibacterial enzymes, such as cathepsin, defensin, lactoferrin, and lysozyme, through degranulation or digestion of pathogens using the same set of enzymes after phagocytosis [12]. Meanwhile, reactive oxygen species (ROS) derived from NADPH oxidases and mitochondria in neutrophils are also toxic to most pathogens [9]. Recently, an unprecedented mechanism called NETosis was proposed as a new killing mechanism of neutrophils. Neutrophil extracellular traps (NETs), consisting of nuclear DNA, histones, proteins, and enzymes from neutrophils, not only immobilize invading pathogens in the circulation to prevent the spread of infection, but also increase the release of antimicrobial histones and proteases to kill pathogens [13,14]. The underlying mechanisms of NETs formation, as well as NETs functions, remain largely unknown, thus warrant future studies.

The death of neutrophils is mediated by cytokines and chemokines released during inflammation. Most neutrophils start apoptosis at the end of their lifespan or become senescent after fighting against pathogens, mediated by interleukin (IL)-23, IL-17, and granulocyte colony-stimulating factor (G-CSF) [15,16]. A small portion of circulatory neutrophils is cleared by tissue-

resident leukocytes such as Kupffer cells in the liver [16]. Moreover, neutrophils also die from the formation of NETs probably because of the irreversible release of many cell components, although the specific mechanism is ill-defined [17–19]. However, not all neutrophils are cleared in the circulation. Some neutrophils reversibly transmigrate back to the bone marrow before being eliminated with the help of CXCR4 on the cell membrane [1,20].

1.1.2 Neutrophil recruitment

1.1.2.1 Classical paradigm

In most cases, in order to fight against pathogens invading into tissues, circulatory neutrophils need to exit the blood flow, leave the venule and migrate to inflammatory sites. This whole process, which is called neutrophil recruitment, starts with tethering of neutrophils and endothelial cells, followed in sequence by neutrophil slowing rolling, firm adhesion, intraluminal crawling, transendothelial migration and interstitial chemotaxis [21]. After stimulation with inflammatory mediators such as histamine, leukotrienes, and cytokines, the starting signal is elicited in endothelial cells, on the surface of which P-selectin and E-selectin are upregulated [22,23]. The P-selectin and E-selectin bind to glycosylated ligands on neutrophils, forming neutrophil-endothelial cell tethering on the rolling neutrophils [22,24]. In addition, lymphocyte function-associated antigen 1 (LFA-1) expressed on neutrophils binds to intercellular adhesion molecule 1 (ICAM-1) on endothelial cells, slowing down the rolling neutrophils and finally promoting the firm adhesion of rolling neutrophils [24]. Meanwhile, neutrophils are activated during the slow-rolling by contacting various chemokines and pathogen-associated molecular patterns (PAMPs) on the surface of the endothelium [1]. Notably, not all neutrophil adhesion requires selectins or integrins. For example, neutrophils adhere to sinusoidal capillaries in the liver through CD44-dependent binding in addition to the binding of MAC-1 and ICAM-1 [25,26]. The CD44-dependent binding is even more important in non-sterile liver inflammation when IL-10 released by Th2 cells inhibits the binding of MAC-1 and ICAM-1 [27].

The adherent neutrophils elongate their cell bodies and protrude their pseudopods, crawling to probe for ideal sites to initiate the transmigration. This intraluminal crawling, directed by small GTPases VAV1 and CDC42, is always perpendicular to the direction of the blood flow and independent of the chemokine gradient [28]. After arriving at the ideal sites, neutrophils transmigrate through the endothelium *via* the paracellular pathway or transcellular pathway. Previous studies revealed that the paracellular pathway was preferred by the transmigrating neutrophils, especially at the tricellular junctions where the endothelial cells had less well-ordered junctional proteins [29,30]. Compared to the paracellular pathway, the transcellular pathway is much slower and less efficient. The endothelial cells that transport neutrophils need to form microvilli-like projections and transmigratory cups. These structures, also termed "endothelial domes", facilitate the transportation of neutrophils in endothelial cells and the attachment of neutrophils to the basement membrane [31,32], where the recruited neutrophils further search for the spot with the least resistance to pass through [33,34].

Several signaling pathways orchestrate the whole process of neutrophil recruitment. Before arriving in the interstitial tissues, neutrophils are directed by the intermediary chemoattractants such as IL-8, leukotriene B_4 (LTB_4), and CXC chemokines released from or presented on endothelial cells. Most of the intermediary chemoattractants bind to G-protein coupled receptors on the surface of neutrophils and activate the phosphoinositide 3-kinase (PI3K) signaling pathway. However, in the interstitial tissues, the PI3K signals elicited by intermediary chemoattractants are overridden by mitogen-activated protein kinase (MAPK) signals elicited by end-target chemoattractants such as bacterial peptide fMLP and complement C5a [35,36]. This signaling hierarchy in neutrophil directional migration suggests that the mechanisms of neutrophil recruitment are specific to stimuli rather than tissues.

1.1.2.2 Reverse transmigration

As we mentioned in the previous section, most of the recruited neutrophils initiate apoptosis after fighting against infections. However, reverse migration, a newly revealed fate for recruited

neutrophils, has been described in recent years. The reverse migration includes reverse luminal crawling (rLC), reverse interstitial migration (rIM), reverse transendothelial cell migration (rTEM) and reverse abluminal crawling (rAC), each of which has distinct mechanisms and physiological/pathophysiological relevance [37]. The rLC refers to the crawling of neutrophils in the opposite direction to that of blood flow *in vivo*. This movement of neutrophils, similar to the classical intraluminal crawling in the venule, is regarded as integrin-mediated leukocyte attachment for the forthcoming migration [38]. The rIM refers to the dispersion of neutrophils away from the foci of the inflammatory sites within interstitial tissues. This inflammation-relieving process is driven by hypoxia inducible factor (HIF) and mediated by multiple mechanisms: 1) desensitization of chemokine signal, 2) elevation of repellent chemokine concentration, 3) elimination of directional cues in the tissues, 4) elevation of repulsive molecules and 5) enhancement of macrophage-mediated repulsion [39–44]. The rTEM refers to the migration through the endothelial cell layer in an abluminal-to-luminal direction. The sites for rTEM are dependent on the integrity of the endothelial junctions which are influenced by the expression of junction adhesion molecule C precursor (JAM-C) [30,45]. The rTEM, together with rAC that refers to the retrograde motility of neutrophils on the pericytes outside the venule wall away from the exit points with unknown mechanisms, posed huge detrimental pathological effects, as they disseminate localized inflammatory responses to secondary organs and cause remote organ damages [30,45,46].

1.1.3 Neutrophil recruitment and diseases

The fact that neutrophils have dual effects on tissues and organs during the inflammation has only been accepted within the last decade. As the first-line defensive cells in innate immunity, neutrophils react rapidly to the pathogens and elicit inflammatory responses to clear the invaders. However, as the clinical relevance of sterile inflammation is gradually revealed, the pro-inflammatory role of neutrophils shows more detrimental effects on tissues and organs with sterile inflammation [47,48].

1.1.3.1 Atherosclerosis

Neutrophils were traditionally not considered to play a role in the formation and progression of atherosclerosis, because of a short cell life span, high turn-over, phenotypic plasticity in tissues and lack of identification in the lesions [49]. However, a recent paper reported that the signal of Ly-6G, a specific mouse neutrophil surface marker, was observed in atherosclerotic lesions in apolipoprotein $E^{-/-}$ ($apoE^{-/-}$) mice [50,51]. Depletion of neutrophils by Ly-6G antibodies significantly decreased the lesion burden in $apoE^{-/-}$ mice [51]. These findings suggest an unprecedented role for recruited neutrophils in the initial stage of atherosclerosis in mice. Moreover, in human atherosclerotic lesions, the expression of CD66b, a human neutrophil marker, is positively related to the occurrence of intraplaque hemorrhage [52,53], indicating neutrophils may also participate in the final stage of atherosclerosis.

1.1.3.2 CNS diseases

The role of recruited neutrophils was also reported in diseases of the central nervous system. Amyloid β peptides, accumulated in patients with Alzheimer's disease, increase neutrophil infiltration into the brain through overexpressing adhesion molecule LFA1 on the surface of circulatory neutrophils. LFA1-mediated neutrophil extravasation further increases the level of IL-17 in brain tissue, aggravating the cognitive loss in these patients [54,55]. In addition, increased neutrophil recruitment and vascular permeability were found in the epilepsy mouse model [56]. Depletion of neutrophils decreased acute and recurrent seizures in mice, suggesting that neutrophil recruitment may contribute to the symptoms of epilepsy in mice albeit with unknown mechanisms [57].

1.1.3.3 Obesity, diabetes, and wound healing

Neutrophil recruitment is increased in obese patients and, in return, the recruited neutrophils further exacerbate obesity in these patients. The infiltrated neutrophils in adipose tissues release a large amount of elastase. The overexpressed elastase degrades insulin receptor substrate 1, shutting

down the energy consumption mediated by insulin [58]. Meanwhile, the recruited neutrophils also reduce β-oxidation of fatty acids and lower energy expenditure in obese patients [59]. This neutrophil-mediated non-resolving sterile inflammation lays the pathological foundation for type II diabetes caused by insulin resistance and its complications such as delayed wound healing. Under physiological conditions, the recruited neutrophils clear areas for tissue rebuilding by digesting extracellular matrix and intracellular matrix from damaged cells as well as releasing VEGF-A for revascularization [60,61]. However, in patients with obesity and diabetes, a high level of glucose stimulates recruited neutrophils to perform NETosis which further delays wound healing and tissue repair [58].

1.1.3.4 Cancer

As a cell type with a short life span, neutrophils are unexpectedly involved, as a "double-edged sword", in the initiation, growth, and metastatic spread of tumors. The involvement of neutrophil recruitment in tumorigenesis was confirmed when CXCR2 ligands were enriched in patients and mice with colitis-associated tumor. Inhibition of CXCR2 or depletion of neutrophils decreased the chances of tumorigenesis [62,63]. Moreover, interleukin-8, a crucial CXCR2 ligand in human, binds to epidermal growth factor receptors to promote cell proliferation in non-small cell lung cancer [64]. The underlying mechanisms for neutrophil-mediated tumorigenesis remain largely unknown except for the disruption of cellular DNA caused by neutrophilic ROS/RNS [65,66] and the increased angiogenesis caused by neutrophil enzymes matrix metallopeptidase 9 (MMP9) and elastase [67,68].

On the contrary, neutrophils also have an inhibitory role for the growth of tumors. Purified neutrophils were reported to kill tumor cells in a study 30 years ago [69]. The depletion of neutrophils promotes tumor growth in mouse models, further strengthening this long-existing assertion [70,71]. As far as we know, neutrophils may not perform this role directly but through releasing cytokines to enhance T cell responses in the clearance of tumor cells [72]. However, previous studies regarding cell signaling in neutrophils indicated that neutrophils might also have

a role in tumorigenesis because stimulation with IL-17 and G-CSF changes some neutrophil phenotypes from cytotoxic to pro-angiogenic [73]. Given that neutrophils release MMP9 that increases the expression of VEGF and fibroblast growth factor 2 (FGF2) [74], the strong pro-angiogenic role of recruited neutrophils might facilitate tumor growth in animal models and patients.

The role of neutrophils in tumor metastasis is a controversial topic as well. Many previous studies revealed that recruited neutrophils might promote metastasis through suppressing the activation of $CD8^+$ T cells and NK cells, increasing NETs formation, and releasing leukotrienes [75–78]. On the contrary, a previous study proposed that neutrophils might kill tumor cells at the pre-metastasis sites [79].

1.2 Microvascular permeability—the passage of the barrier between adherent neutrophils and the interstitial inflammatory site

In acute inflammation, one of the core events is the interaction between the recruited neutrophils and endothelial cells. This interaction elicits signaling changes in both cell types and further impairs the endothelial barrier function by increasing adjacent endothelial spaces. Specifically, the neutrophil-endothelium interaction increases microvascular permeability *via* three mechanisms: 1) secreted neutrophil products, 2) neutrophil adhesion and transmigration, and 3) local respiratory bursts, such as the production of ROS and RNS [80].

1.2.1 Secreted neutrophil products and microvascular permeability

Neutrophils are partially activated when they merely receive signals through ligands for G-protein coupled receptors [81], integrins [82], P-selectin glycoprotein ligand 1 (PSGL-1) or L-selectins [83]. Full activation of neutrophils is only achieved when respiratory burst and degranulation are also involved [84]. In either situation, activation of neutrophils releases soluble factors that bind receptors on endothelial cells. This binding further increases calcium influx into endothelial cells and activates myosin light chain kinases (MLCK) that phosphorylate myosin. The

phosphorylation of myosin elevates microvascular permeability through inducing EC contraction [85,86]. Meanwhile, the increased intracellular calcium level also activates small GTPases RhoA and ROCK *via* activation of PKCα in endothelial cells. RhoA and ROCK further trigger endothelial cell contraction and increase microvascular permeability by changing the actin polymerization of endothelial cells [85,86]. Moreover, some soluble factors directly damage the barrier function of endothelium by causing apoptosis of endothelial cells or VE-cadherin-mediated opening of intercellular junctions between endothelial cells [86].

Neutrophilic intracellular enzymes, such as elastase and cathepsin G, are bactericidal and cytotoxic mediators released from neutrophilic primary (azurophilic) granules when neutrophils are appropriately activated. These enzymes digest VE-cadherin, a key component of adherens junctions in the endothelium which prevents the leakage of large molecules such as albumin. However, the impairment of endothelium by elastase and cathepsin G increases microvascular permeability without changing neutrophil recruitment, probably because they avoid simultaneously influencing other signals in endothelial cells [87–91]. Other intracellular enzymes, such as cytosolic phospholipase, 5-lipoxygenase and cyclooxygenase, also regulate the microvascular barrier function, which is evidenced by previous studies that utilized gene modifications to suppress the activation of the enzymes and observed decreased microvascular leakage in LPS/zymosan-induced mouse acute lung injuries [92–94].

Arachidonic acid derivatives are another class of important inflammatory mediators released by leukocytes. Unlike most leukocytes that synthesize and release prostaglandins, neutrophils synthesize and release leukotrienes during acute inflammation [95]. Interestingly, LTB_4 alone failed to induce microvascular hyperpermeability in fMLP-treated mouse models, whereas the addition of PGE_2 to LTB_4 dramatically increased microvascular permeability [96]. This might be because LTB_4 decreased blood flow in the microcirculation and PGE_2 relaxed pre-capillary arterioles to facilitate the measurement of leakage. Moreover, LTB_4 was reported to promote adhesion-mediated microvascular hyperpermeability [97,98]. In addition, LTB_4 also increases microvascular leakage indirectly through increasing the release of neutrophilic heparin-binding protein (HBP), a fast-

released protein from neutrophils in acute inflammation [99]. The HBP binds to the receptors for prostaglandins on endothelial cells and causes endothelial cell contraction through triggering calcium influx [100]. Meanwhile, the increased calcium level in endothelial cells results in the internalization of VE-cadherin, ending up with the opening of adherens junctions of the endothelium [101]. Besides LTB_4, LTC_4 and LTD_4 directly bind to their receptors on endothelial cells and increase microvascular permeability by causing endothelial cell contraction [102,103].

The chemokines such as CXCL2 and CXCL8 are not stored for a long time in neutrophils but synthesized by many different cell types [104,105]. These inflammatory mediators increase microvascular permeability by distinct mechanisms: 1) TNFα activates PKC and p38 MAPK and increases microtubule destabilization, which increases the formation of intracellular gaps [106,107]; 2) CXC chemokines are prone to induce microvascular hyperpermeability through promoting angiogenesis and increasing leukocyte transmigration [108,109]; 3) CXCL8, on the other side, activates Rac/PAK to increase the phosphorylation and internalization of VE-cadherin, which opens the adherens junctions of endothelium [110].

Furthermore, activation of some biological molecules such as complements C3a, C4a, and C5a regulates microvascular permeability through binding the complement receptors on endothelial cells and triggering cell contraction [111–113]. Angiopoietin 2 released from innate and adaptive immune cells also increases microvascular leakage by inducing apoptosis of endothelial cells and enhancing leukocyte adhesion [114–116].

1.2.2 Neutrophil adhesion and microvascular permeability

In addition to neutrophil-derived soluble factors, firm adhesion of neutrophils to endothelium mediated by endothelial adhesion molecules ICAM-1, VCAM-1 and E-selectin regulates microvascular permeability through triggering calcium-mediated endothelial cell contraction [117]. ICAM-1-mediated calcium influx activates endothelial actin polymerization and further destabilizes endothelial barrier by reorganizing the cytoskeleton of endothelial cells [117]. By contrast, ligation of VCAM-1 not only shares a similar mechanism in disrupting the barrier function

[117] but also is required in the synthesis of NADPH oxidase in neutrophils, an enzyme that synthesizes hydrogen peroxide which impairs endothelial barrier function [118,119]. However, VCAM-1 has less clinical significance than ICAM-1 in regulating microcirculation, probably because the binding of VCAM-1 and integrin $\alpha_4\beta_1$ is not expressed in human neutrophils [120]. Moreover, ligation of E-selectin induced association of cytoskeletal proteins (α-actinin, vinculin, filamin, FAK, and paxillin) with the cytoplasmic domain of E-selectin to reshape the endothelial cells and cause contraction of endothelial cells [117].

Besides the endothelial cell contraction that interrupts the barrier function, firm adhesion caused by ligation of adhesion molecules also opens adherens junctions between neighboring endothelial cells to promote microvascular leakage. Intracellular signals triggered by ICAM-1 ligation dissociate VE-protein tyrosine phosphate (PTP) from VE-cadherin by preventing the binding of β-catenin to VE-cadherin in endothelial cells. This induces the phosphorylation of VE-cadherin, which further results in the internalization of VE-cadherin and opens the adherens junctions allowing the leakage of large proteins from microvasculature [121,122]. The transcytosis pathway is another mechanism by which adhesion molecules modulate microvascular permeability. It was reported that ICAM-1-mediated increased Src phosphorylation of caveolin-1 significantly enhances caveolae-dependent transcytosis of labeled albumin *in vivo* and *in vitro* [123].

1.2.3 Neutrophil transmigration and microvascular permeability

The role of neutrophil transmigration in regulating microvascular leakage is still a controversy. Previous studies showed that firm adhesion of neutrophils without transmigration was sufficient to increase microvascular leakage [124,125], whereas in other cases adhesion alone failed to do so [32,98], suggesting that neutrophil transmigration may contribute to the elevation of microvascular permeability. It was believed for a long time that microvascular leakage was due to temporary openings created by transmigrating leukocytes [124]. However, recent studies showed an inversely protective role for neutrophil transmigration in modulating the barrier function of endothelial cells. When the expression of ICAM-1 in endothelial cells was high, adherent

neutrophils preferred to transmigrate through the transcellular route rather than the paracellular route, with the help of newly formed “endothelial domes” [23,126]. Similarly, the binding of LFA-1 and ICAM-1 in firm adhesion and transmigration were redistributed to the sites at the junctions of endothelial cells, forming some “ring-like structures” [122,127]. These “domes” and “ring-like structures” caused by neutrophil transmigration actually minimized the microvascular leakage, implying that neutrophil transmigration may play an important role in maintaining the integrity of endothelium [32].

1.2.4 Neutrophil ROS and microvascular permeability

Excessive generation of ROS by activated neutrophils has been implicated as having comprehensive toxic effects on the barrier function of the endothelium [128]. Firstly, the production of ROS increases the expression of cell surface adhesion molecules on endothelial cells, elevating microvascular leakage through the increased leukocyte firm adhesion [129]. Secondly, neutrophilic ROS production activates protein kinases and phosphatases in endothelial cells. This activation further dissociates β-catenin from VE-cadherin, resulting in the opening of adherens junctions through VE-cadherin internalization [130]. Meanwhile, the activation of protein kinases and phosphatases in endothelial cells also activates actin polymerization and causes cytoskeletal remodeling, which is the key step in endothelial cell contraction [131]. Thirdly, extracellular ROS released by neutrophils enters intracellular gaps between endothelial cells, causing the redistribution of tight junction transmembrane protein. The disruption of tight junctions impairs the integrity of the endothelium and increases the leakage of small molecules [132]. Interestingly, the substantial studies that have revealed the toxic effects of neutrophilic ROS and the beneficial effects of antioxidants notwithstanding, applications of antioxidants in clinical trials showed significantly lower efficacy than those in animal models in preventing microvascular hyperpermeability caused by inflammatory stimulants [133,134]. This might be because the patients enrolled in these studies had already been overloaded with oxidative burden before they had clinical symptoms, and thus the timing of giving antioxidant therapies might be too late to

reverse the damage caused by neutrophilic ROS in the endothelium. Moreover, the limited therapeutic functions of some oral antioxidants may also be because of the dependence on the patient specific metabolic demand and genetic background [135].

1.2.5 Enhancers for microvascular barrier function

In addition to the "domes" and "ring-like structures" formed during neutrophil transmigration, a continuous-growing list of small molecules in microcirculation are identified as enhancers for the endothelial barrier function. Sphingosine 1-phosphate (S1P), released by erythrocytes and platelets, binds to 5 subtypes of receptors (S1PR1–S1PR5) on endothelial cells [136]. A physiological level (5–40 μmol/L in blood) of S1P preferably binds to S1P1R and S1P2R which increases the expression of VE-cadherin and β-catenin through activation of small GTPases Rac1/ROCK [137]. However, pathologically high levels of S1P preferably binds to S1P3R on endothelial cells, resulting in the disruption of endothelial barrier function through Rho-dependent signals [137]. Moreover, adenosine, an extracellularly released nucleotide accumulated during inflammation, shows an opposite role that protects the microvascular barrier function through binding to the A2b receptor on endothelial cells [138–140]. CD47, an immunoglobulin superfamily transmembrane glycoprotein, also performs a protective role in microvascular permeability. This was evidenced by a previous study observing increased neutrophil recruitment as well as microvascular leakage in mice challenged by LPS and *E. coli* after depletion of CD47, albeit the underlying mechanism remained unclear [141].

1.3 Reactive oxygen species—a powerful weapon of recruited neutrophils

Reactive oxygen species (ROS) are a group of chemically reactive species containing oxygen. They can be divided into two subcategories—radicals and non-radicals, based on whether they possess one or more unpaired electrons and are capable of independent existence [142]. For example, superoxide is a typical radical that has one unpaired electron in the ROS family, whereas hydrogen peroxide is classified as a non-radical but still a member of the ROS family [143]. The

ROS, as strong oxidants, participate in various biological reactions. Under physiological conditions, the ROS conduct intracellular and extracellular signals when the concentration is low. However, certain pathological processes significantly increase the levels of ROS, causing irreversible oxidative stress-mediated damage in different organs and tissues [144,145].

1.3.1 Sources

The majority of ROS are produced from four intracellular sources. The first and most important source is mitochondria, in which two electron transport chains (ETC) — complex I (NADH dehydrogenase) and complex III (ubiquinone cytochrome c reductase) — are involved. Because the ETC-derived ROS generation is non-enzymatic, the production of ROS in the mitochondria will be increased along with the increased metabolic status [146]. Another main source for ROS is the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase bound on the cell membrane. This complex, consisting of six subunits, faces the extracellular space and is activated for producing both intracellular and extracellular ROS in many leukocytes [147]. Moreover, peroxisomes contribute to the production of hydrogen peroxide through the β-oxidation of fatty acids [148]. Endoplasmic reticulum also contains enzymes, such as cytochrome P450, b5 enzyme, and diamine oxidase, which are required for the production of ROS in addition to the sources mentioned previously [149].

1.3.2 Targets

ROS interrupt the biological functions of almost all cellular components. They react with the purine and pyrimidine within DNA, breaking DNA double strands [150]. Similarly, RNA is even more sensitive to ROS, because of the structure of single strand and lack of protection by proteins [151]. ROS also oxidize membrane lipids, such as phospholipids [152]. This oxidation of phospholipids reduces the membrane fluidity and inactivates the membrane-bound enzymes and receptors. Moreover, ROS disrupt the functions of cellular proteins through oxidizing sulfur-containing amino acids such as methionine and cysteine [153]. The oxidation of the reducing amino

acids increases the protein-protein cross-linking, resulting in the loss of functions of the sulfur-containing proteins [154,155].

1.3.3 ROS and diseases

1.3.3.1 Diabetes mellitus

Although ROS are produced by normal metabolism, the imbalance of antioxidants and oxidants caused by the increasing production of ROS contribute to many diseases. Diabetes mellitus (DM) is a heterogeneous group of chronic disorders resulting from defective insulin secretion or resistance to insulin [156]. The increased generation of local ROS, which contributes to the pathogenesis of DM complications, is caused by the elevated level of blood glucose in patients with DM through four mechanisms: 1) redox imbalance from the increased glycolysis, 2) decreased glutathione from the activated sorbitol pathway, 3) auto-oxidation of glucose and 4) non-enzymatic protein glycation [157].

1.3.3.2 Neurodegenerative diseases

Elevated ROS is prevalent in many neurodegenerative diseases, albeit the sources of the ROS are still poorly defined. In patients with Parkinson's disease, the increased local ROS directly damage dopamine neurons, disrupting the synthesis and metabolism of dopamine in the brain. In turn, dopamine, as a metal chelator, increases the production of hydrogen peroxide through Fenton reactions *in vivo* [158]. In Alzheimer's disease, the accumulated local oxidative stress comes from amyloid β peptides which chelate with transition metal ions. This increased level of toxic radicals induces lipid peroxidation and promotes neuron death by blocking the biological functions of ion pumps, glucose transporters, and glutamate transporters [159]. Similarly, the lipid peroxidation caused by the toxic radicals also results in neuron demyelination, the core pathogenesis for multiple sclerosis [160].

1.3.3.3 Cardiovascular diseases

The notorious roles for ROS in the development of atherosclerosis and hypertension have been reported by many previous studies. The ROS, generated by endothelial cells and circulatory leukocytes, cause injury and dysfunction of endothelial cells, further promoting the progress of atherosclerotic lesions [161]. Meanwhile, the ROS, together with eNOS-derived nitric oxide (NO), inhibit cell proliferation and facilitate cell apoptosis in the vasculature by interrupting intracellular DNA repair [162,163]. In hypertension, the increased ROS continuously consume NO, a vasodilator, and generate peroxynitrite ($ONOO^-$), a vasoconstrictor. The imbalance of NO and $ONOO^-$ disrupts the endothelium-dependent vasodilation, further raising blood pressure by increasing total peripheral resistance [164,165].

1.3.3.4 Autoimmune diseases

Increased production of ROS exacerbates inflammation-related symptoms of many autoimmune diseases because of their toxicity to normal tissues. In rheumatoid arthritis, the excessive ROS damage cartilage in the joints *via* increasing the production of MMP9, an enzyme that suppresses the formation of matrix components [166,167]. In cataracts, ROS increase the oxidation of cellular proteins, lipids, and DNA, resulting in lens opacification in patients [168]. Moreover, the production of ROS in asthma increases the hyper-responsiveness of the airways by inducing the production of inflammatory mediators from macrophages, neutrophils, and eosinophils [169,170].

1.3.3.5 Cancer

As compared with normal cells, tumor cells produce higher levels of ROS and are more sensitive to mitochondrial dysfunction. Firstly, cigarette smoking is one of the most important risk factors in the carcinogenesis of lung cancer, because the burning of cigarette components produces stable ROS that have extremely long half-lives. The stable ROS increase the transformation of cells into malignant forms, resulting in the initiation of lung tumors [171]. Secondly, ROS produced by the increased thymidine phosphorylation in breast cancer tissues induce hyperplasia of the

epithelium, one type of precancerous lesion [172]. Third, NOX1 (a subunit of the NADPH oxidase) - derived ROS in prostate cancer disrupt normal cellular immortality [173]. Moreover, the increased ROS production promotes not only the initiation of tumors but also the metastasis. The increased oxidative stress in bladder cancer stimulates the increased production of MMP9, facilitating tumor spreading through breaking down collagen in tissue [174].

1.4 CXCL2 and WKYMVm—important chemoattractants for neutrophil recruitment and activation

1.4.1 CXCL2—a classical "intermediary" chemoattractant

CXCL2, also called macrophage inflammatory protein-2 (MIP-2) and growth-related oncogene-b (GROb), is a ELR-CXC chemokine (a chemokine containing a glutamate-leucine-arginine motif immediately before the amino-terminal CXC motif) that triggers neutrophil chemotaxis and activation [175,176]. CXCL2 is structurally closely to its homologue CXCL1, which is also known as keratinocyte chemoattractant (KC) in mice, with the only differences at two conserved basic residues in their sequences [177]. CXCL2 acts on both the chemokine receptor 1 (CXCR1) and chemokine receptor 2 (CXCR2) but has higher affinity to CXCR2 to boost mouse neutrophil migration, whereas CXCL1 has comparatively low affinity to CXCR2 and may function in priming circulatory neutrophils [21].

CXCL2 is released by a wide range of cell types, however, the regulation of CXCL2 production in different cells varies. Firstly, the release of CXCL2 in macrophages and monocytes is regulated by the activation of surface Toll-like receptors through adaptor proteins MyD88 and TRIF. Although it has been shown that single involvement of either pathway is sufficient to trigger an influx of neutrophils, the use of both pathways results in maximal production of CXCL2 from macrophages. Secondly, in liver sterile inflammation, the release of CXCL2 in injured lobes increases 100–1000-fold [178], which is caused by Fas-induced activation of caspase-3 and the nuclear translocation of activator protein-1 [179]. Moreover, the hepatic CXCL2 production induced by bile acids can also be regulated through IL-17A and IL-10 [180–182]. Thirdly, the

expression of CXCL2 in epithelial cells in LPS- or IL-1β-treated rat small intestine is regulated by the level of histone acetylase *in vivo* [183].

As one of the important intermediary chemoattractants, CXCL2 participates in orchestrating neutrophil recruitment at very early stages. The elevation of CXCL2 on the surface of liver sinusoids surround sites of necrosis occurs in the early phases of tissue reperfusion, earlier than any observable neutrophil recruitment [47,184]. This indicates that CXCL2 may play a dominant role in the initiation of neutrophil infiltration in liver inflammation. Moreover, knocking-out CXCR2 in mice failed to completely block but decreased the strength and delayed the timing of neutrophil recruitment, suggesting that CXCL2, albeit important, is not the only chemoattractant that drives neutrophil recruitment [185,186]. Later, CXCL2 is reportedly only able to stimulate a certain type of neutrophil population called pro-inflammatory neutrophils ($CD11b^{+}Gr\text{-}1^{+}CXCR4^{low}$), whereas VEGF-A is responsible for promoting the recruitment of pro-angiogenic neutrophils which express MMP9 and CXCR4 [60].

In addition to the sterile inflammation in the liver, CXCL2 is also required in neutrophil recruitment in other diseases. This is evidenced by previous studies showing that CXCL2 increases neutrophil recruitment in the inflammation of rat lungs as well as mouse intestines [187,188], whereas the inhibition of CXCL2 by specific antibodies decreases neutrophil recruitment [189,190]. Furthermore, the elevated infiltration of neutrophils induced by CXCL2 is positively correlated to the severity of keratitis, a corneal disease caused by *Staphylococcus aureus* [191]. In allergy, the secretion of CXCL1/2 or CXCL8 results in neutrophil recruitment to the airway in TLR4-mediated, pollen allergen- or cat dander-induced airway hypersensitivity [192]. In neoplastic diseases, CXCL1/2 promotes the recruitment of $S100SA8/9^{+}$ neutrophils which increase the survival of tumor cells and their resistance to chemotherapy [193].

1.4.2 fMLP and WKYMVm—formylated peptides function as "end-target" chemoattractants

The synthesis of bacterial proteins starts with a formylated methionine (fMet), which is subsequentially cleaved off during the growth of bacteria. In 1970s, this fMet in the N-terminus of bacterial peptides was found recognizable by cells from the human innate immune system, such as macrophages and neutrophils [194,195]. N-Formylmethionine-leucyl-phenylalanine (fMLP), one of the fMet-containing peptides originated from bacteria, and its five-peptide mimic Trp-Lys-Tyr-Met-Val-L-Met (WKYMVm) are known as potent chemoattractants and activators for neutrophils in the directional migration, mobilization of receptors, and release of proteolytic enzymes.

Neutrophils respond to fMLP and WKYMVm through formyl peptide receptors (FPRs), which are G-protein coupled receptors (GPCRs) originally stored in the granules and transferred onto the surface of cell membranes after stimulation with priming agents or during the neutrophil transmigration process. Human neutrophils have three types of formyl peptide receptors (hFPR1, hFPR2 and hFPR3), whereas mice have at least seven types of formyl peptide receptors (mFpr1, mFpr-rs1, mFpr2, mFpr-rs3, mFpr-rs4, mFpr-rs6 and mFpr-rs7). The mFprs have over 50% amino acid sequence identity with each other and with the three hFPRs. Among these receptors, mFpr2 and mFpr-rs1 share many properties with hFPR2, except that they have little affinity to fMLP. In humans, WKYMVm binds to hFPR2, but also has weak effects on hFPR1 and hFPR3. Similarly, in mouse, WKYMVm preferentially stimulates mFpr2, but its effects on other six mouse mFprs cannot be ruled out. Both hFPRs and mFprs have high identity in the overall amino acid sequence in cytosolic parts, whereas lower identities in the extracellular domains. This implies that the differences among hFPRs or mFprs exist in ligand binding rather than downstream intracellular signaling pathway. The binding of fMLP or WKYMVm to FPRs thereafter activates $G\alpha_i\beta_\gamma$ and downstream phospholipases and protein kinases. Other than in myeloid cells such as neutrophils and macrophages, FPRs are also widely expressed in non-myeloid cells such as natural killer cells, smooth muscle cells, endothelial cells and even in cells of central nervous system [196,197]. However, FPRs have not been found in red blood cells, lymphocytes, and platelets [198].

The activation of FPRs initiates many fMLP- or WKYMVm-induced neutrophil responses, one of which is the chemotactic migration of neutrophils. Human FPRs were reported to contribute

to the detection of chemotactic gradient in neutrophils [199,200]. Interestingly, a higher level of chemoattractant does not always guarantee a stronger chemotactic response. This is evidenced by the study showing a concentration below the detection limit will blunt the chemotaxis of neutrophils [201]. It may be because saturation of the receptors diminishes any meaningful gradients or because of desensitization of the receptors themselves. After the binding of fMLP or WKYMVm to FPRs, neutrophils undergo a stereotyped progression to rearrange the cytoskeleton in a short time period through Rac-mediated actin polymerization [202]. Neutrophil chemotaxis depends very little on the internalization or redistribution of FPRs to the leading edge, only requiring the release of $G\beta_{\gamma}$ from the activated $G\alpha_i$ [203,204].

Another important response mediated by FPRs in neutrophils is the superoxide generation. Unlike neutrophil chemotaxis induced by low level of fMLP, the generation of superoxide normally requires a very high concentration of fMLP [205,206]. This dose-dependent pattern, probably due to the requirement for high intracellular signaling strength in NADPH oxidase activation in neutrophils, minimizes oxidant-mediated tissue injury caused by low fMLP concentrations during neutrophil transmigration. In addition, although being unable to stimulate the generation of superoxide, low level of fMLP can prime neutrophils for a more robust "respiratory burst" to other stimulants [207].

fMLP and WKYMVm also induce degranulation, transcriptional regulation, and apoptosis in neutrophils through FPRs. fMLP increases the release of granule constituents from neutrophils in a dose-dependent manner. A lower dose of fMLP first promotes the release of secretory vesicles and gelatinase granules, followed by the massive release of other granules at a higher dose of fMLP [208]. Moreover, fMLP regulates the synthesis of proinflammatory cytokines through modulating the expression of nuclear transcription factors [209,210]. In addition, unlike G-CSF and IL-1β that increase the half-life of neutrophils, fMLP shortens the half-life by inducing neutrophil apoptosis through FPRs [7,211].

1.5 PI3K and MAPK—intracellular signaling pathways from membrane receptors to the downstream effectors

The recruitment and activation of neutrophils is a multi-step process orchestrated by various factors during acute inflammation. Two key components in this process are the establishment of the appropriate chemoattractant gradient and the intrinsic ability of the neutrophils to migrate along the gradients. The previous section introduced the distinctive effects of the intermediary chemoattractant and the "end-target" chemoattractant on mediating neutrophil recruitment and activation. Here, I would further introduce the intrinsic signaling pathways that contribute to the gradient sensation of neutrophils, the polarization of neutrophils, the upregulation of neutrophil surface adhesion molecules, and the generation of neutrophil products. Considering that the receptors for the stimuli throughout my thesis are GPCRs (CXCR2 for the chemokine CXCL2 and formyl peptide receptors for the formyl peptide receptor agonist WKYMVm), I will only focus on introducing the PI3K signaling pathway and MAPK signaling pathway, two main pathways downstream of GPCR in the translation of extracellular chemoattractant binding to the directed cellular movements and activated cellular responses.

1.5.1 PI3K signaling pathway

PI3K is a family of structurally and functionally related intracellular kinases regulating many cellular functions. It can be divided into three subfamilies and eight isoforms: Class I (PI3Kα, β, γ, δ), Class II (PI3KC2α, C2β, C2γ), and Class III PI3K [212,213]. Class I PI3Ks selectively phosphorylate phosphatidylinositol 4,5-biphosphate (PI(4,5)P2, also known as PIP2) to form phosphatidylinositol 3,4,5-triphosphate (PI(3,4,5)P3, also known as PIP3), while Class II and Class III PI3Ks selectively phosphorylate phosphatidylinositol (PI) to generate phosphatidylinositol 3-phosphate (PI3P) [214].

Each PI3K isoform has its own regulatory subunits that control the activation of the enzyme and catalytic subunits that perform enzymatic functions. The PI3K regulatory subunits increase the activity of membrane-bound small GTPases after the binding of extracellular ligands and cell

surface receptors. The activated small GTPases in the GTP-bound form activate the PI3K catalytic subunits to elevate the generation of substrates PIP2 and PIP3 on the cell membrane [215]. These PIP2 and PIP3 further bind pleckstrin homology (PH) domain-containing proteins, known as PI3K adaptors and effectors such as Btk and Akt, to perform various biological functions downstream of PI3K signaling pathway [216,217].

PI3K isoforms mediate extensive cellular functions in the immune system: 1) PI3Kα, PI3Kβ, and PI3Kδ, belonging to Class IA PI3K, play key roles in the development of B lymphocytes and T lymphocytes; 2) PI3Kγ, the only member of Class IB PI3K, regulates chemotaxis, recruitment, and activation in leukocytes; 3) Class II PI3Ks (C2α/C2β/C2γ) are required in endocytosis and thrombi formation; 4) Class III PI3K, the latest member in the PI3K family, functions in endosome-lysosome trafficking, autophagy induction, and pathogen killing [218].

1.5.1.1 PI3K isoforms in neutrophil recruitment

Many studies have reported that the isoforms of PI3K, especially PI3Kγ, contribute to almost every step of neutrophil recruitment [43,219–222]. Being downstream of GPCRs, the PI3Kγ-mediated roles in regulating neutrophil recruitment vary from stimulus to stimulus. Although PI3Kγ is not the unique signal in sensing the gradient of chemoattractants, it determines the initiation of neutrophil movement and is critically required in the subsequent directed neutrophil movement along the gradient after stimulation with fMLP. Moreover, PI3Kγ increases integrin-mediated neutrophil adhesion and crawling through increasing the fMLP-induced generation of PIP2/PIP3 [223]. Similarly, PI3Kγ facilitates cell adhesion by strengthening the integrin (LFA-1) bond in neutrophils stimulated with chemokine CXCL1, CXCL2, or complement C5a [224]. Meanwhile, PI3Kγ is also required in chemokine-induced neutrophil chemotaxis because it promotes the tyrosine-phosphorylation of vimentin [225].

In addition to the dominant role of PI3Kγ in neutrophil recruitment, PI3Kδ is co-involved and performs a synergistic role in this process. PI3Kδ mainly assists in the directional “compass” of the neutrophil without changing the assembly of F-actin in neutrophils, suggesting that PI3Kδ

may only function in the neutrophil sensation of chemoattractant gradient but have little effect on cell adhesion and movement [226]. Interestingly, PI3Kδ is believed to play a temporal role. Unlike PI3Kγ that dominates in the early stage of neutrophil recruitment, PI3Kδ may dominate in the late stage [227].

Beyond their roles in neutrophils, PI3Kγ and PI3Kδ also promote neutrophil recruitment through their roles in endothelial cells. PI3Kγ regulates the expression of E-selectin on endothelial cells, further decreasing the E-selectin-mediated neutrophil velocity and increasing neutrophil adhesion [228]. PI3Kδ can also increase neutrophil adhesion by activating Akt-dependent phosphorylation of PDK1 in endothelial cells [229].

1.5.2 MAPK signaling pathway

MAPK is a group of protein Ser/Thr kinases that translate extracellular stimulation into cellular responses. They regulate the processes of gene expression, mitosis, metabolism, motility, survival, apoptosis, and differentiation in most if not all eukaryotic cells [230]. Fourteen types of MAPKs are divided into two categories: 1) the conventional MAPKs that include extracellular signal-regulated kinases 1/2 (ERK1/2), c-Jun N-terminal kinases 1/2/3 (JNK1/2/3), p38 α/β/γ/δ, and ERK5, and 2) the atypical MAPKs that include ERK3/4, ERK7, and nemo-like kinase [231].

MAPKs are activated by their upstream evolutionarily conserved kinases. The membrane bound small GTPases in the Ras and Rho families interact with MAPK kinase kinases, further activating MAPK kinases which sequentially allow the activation of MAPKs. In activated conventional MAPKs the Thr and Tyr residues are dually phosphorylated to form a motif of Thr-X-Tyr located in the activation loop of the kinase to perform functions [232]. However, in the atypical MAPKs, the mechanism of activation is still unknown because of the absence of the formation of the Thr-X-Tyr motif [233].

MAPKs conduct cellular signaling through phosphorylation of their downstream substrates [234,235], also known as MAPK activated protein kinases (MAPKAPK). These activated protein kinases include the p90 ribosomal S6 kinases (RSK) [236], mitogen- and stress-activated kinases

[237], MAPK-interacting kinases [238], MAPK-activated protein kinases 2/3 [239], and MAPK-activated protein kinase 5 (MK5) [240]. In quiescent cells, the MAPKs and the corresponding downstream MAPKAPKs locate in the cytoplasm while, after stimulation, they accumulate in the nucleus and constitute a variety of modules that regulate different biological functions [241,242].

1.5.2.1 MAPKs in neutrophil motility and activation

The major roles of MAPKs reported in a large number of previous studies center on cell proliferation and differentiation. However, MAPKs, especially the conventional MAPKs, can regulate cell protrusion, cell-matrix adhesion, cell retraction, and exocytosis through phosphorylation of the cell's motility machinery. The universal targets of MAPKs enable them to regulate movement in various mobile immune cells and even in tumor cells [243]. G-CSF-induced activation of ERK1/2 in neutrophils results in the phosphorylation of myosin light chain (MLC), allowing the redistribution of F-actin that mediates neutrophil movement [244]. Moreover, the activity of ERK1/2 regulated by protein kinase A (PKA) is positively related to neutrophil recruitment in nonsteroidal anti-inflammatory drug-induced enteritis, probably through regulating neutrophil adhesion [245]. Interestingly, ERK5, another conventional MAPK downstream of MEK, is activated by fMLP, but this activation of MEK/ERK5 dominates only when the dose of fMLP is low. Alternatively, the activation of MEK/ERK1/ERK2 recaptures the dominant role in regulating neutrophil recruitment induced by high doses of fMLP [246]. In addition, JNK is also required in neutrophil recruitment because inhibition of JNK phosphorylation decreases LPS-induced mouse pulmonary neutrophil infiltration *via* downregulating the expression of plasminogen activator inhibitor-1 (PAI-1) [247].

Unlike the supportive roles of ERK and JNK, the role of p38 in neutrophil recruitment is still debatable. TNFα initiates a stop signal in neutrophil polarization by markedly increasing the phosphorylation of p38 but not ERK, suggesting that p38 may mediate a suppressive signal that facilitates neutrophils to remain rather than to migrate [248]. This p38-mediated stoppage signal also exists in LPS-induced neutrophil chemotaxis through a mechanism involving autocrine ATP

signaling-inducing, p38-dependent MLC phosphorylation [249]. However, in KC/CXCL1-induced neutrophil recruitment, p38 MAPK seems to play a supportive role, as it was evidenced by the fact that pharmacological inhibition of p38 decreased the migration velocity and directionality during the neutrophil recruitment [250]. In addition, p38 is critically required in the production of proinflammatory cytokines, suggesting that p38 may promote neutrophil recruitment in an indirect way [66].

Beyond the above-mentioned roles of the conventional MAPKs in neutrophil recruitment and chemotaxis, the MAPKs also contribute to the activation of neutrophils during acute inflammation. In previous studies, p38 was reported to regulate the respiratory burst in TNFα- or LPS-primed neutrophils by increasing the membrane expression of flavocytochrome b_{558} and the exocytosis of intracellular granules [251]. JNK also mediates the signal downstream of fMLP receptors to initiate the respiratory burst and the F-actin polymerization in neutrophils [252].

1.5.3 The signal hierarchy in neutrophil recruitment

The inflammatory site is a complex environment that always contains chemoattractants from different sources, such as vascular endothelium, interstitial cells (macrophages and mast cells), and the infectious agents. The chemoattractants, including chemokines and leukotrienes, released by the endothelium and interstitial cells are regulated as intermediary chemoattractants. These chemoattractants are the first to be sensed and they initiate neutrophil recruitment partly because they are spatially closer to the circulatory neutrophils as compared with the end-target chemoattractants released by the infectious agents, such as exotoxin fMLP. However, when the neutrophil approaches the inflammatory site, it will face a significantly changed environment with decreasing levels of intermediary chemoattractants and increasing levels of end-target chemoattractants and will be forced to “make decisions” for the subsequential directional movement.

To explain the successful arrival of neutrophils to the inflammatory site, a signaling hierarchy in neutrophil recruitment was proposed by a previous study [36]. In that study, the authors showed that different chemoattractants triggered different intracellular signaling pathways and the

pathways worked in a hierarchical manner. The activation of p38 MAPK pathway induced by the end-target chemoattractants overtakes the activation of PI3K pathway induced by intermediary chemoattractants in regulating neutrophil chemotaxis. The intrinsic drive that enables the neutrophil to make such end-target chemoattractant-preferential decisions, albeit not fully understood, may be due to the p38-mediated stop signal [248] or the possible involvement of PTEN [35,253]. Noticeably, although the p38 pathway overweighs the PI3K pathway in regulating neutrophil chemotaxis, the PI3K pathway poses a dominant inhibitory effect on the p38 pathway in the apoptosis of neutrophils and endothelial cells [254,255], indicating that the signaling hierarchy may vary among different neutrophil responses.

1.6 Bam32—an adaptor for PI3K signaling pathway

The pleckstrin homology domain proteins are the intracellular signaling molecules that interact with the lipid second messengers PIP3 and its breakdown product PIP2, resulting in the recruitment of these molecules to the plasma membrane and the regulation of cell growth, proliferation and differentiation [217,256]. B cell adapter molecule of 32 kDa (Bam32), also termed as dual adaptor for phosphotyrosine and 3-phosphoinositides 1 (DAPP1), one of the PI3K adapter proteins, is a PH-domain protein that possesses an N-terminal Src homology 2 (SH2) domain and a C-terminal PH domain. It is recruited to the plasma membrane, binding to the lipid second messengers PI(3,4,5)P3 and PI(3,4)P2 after B cell receptor (BCR)-induced cross-linking. Unlike other PH domain proteins (such as Btk and Grp-1), Bam32 has a high affinity to bind both PI(3,4,5)P3 and PI(3,4)P2, with only a slight PI(3,4)P2 preference [257]. This phenomenon can be attributed to the differences in their X-ray crystal structures that the 5-phosphate group contributed little to the Bam32-PI(1,3,4,5)P4 complex while this group is essential in the Grp-PI(1,3,4,5)P4 and Btk-PI(1,3,4,5)P4 [258,259].

Bam32 is expressed in various cell types in human, mouse, rat, and chicken. It is also expressed in human bone marrow, spleen, lymph node, trachea, placenta, and peripheral blood leukocytes. Among leukocytes, Bam32 is highly expressed in all B cell lines [257]. Later studies revealed that Bam32 is also expressed in T lymphocytes [260], mast cells [261], and dendritic cells

[262]. However, the murine Bam32 is hardly expressed in isolated germinal center B cells or activated splenic B cells. Moreover, low levels of Bam32 mRNA were observed in B cells stimulated with LPS, anti-CD40 and anti-IgM [263].

The membrane recruitment of Bam32 after the BCR-induced activation in B lymphocytes is PI3K-dependent. Tyrosine 139 in Bam32 was phosphorylated by Src family tyrosine kinases, and then the PH domain in Bam32 bound to the PI(3,4,5)P3 and its dephosphorylation product PI(3,4)P2 [264,265]. Although SHIP was a negative regulator in Btk/PLC signaling pathway, its function in the Bam32 pathway was quite the opposite—Bam32 was resistant to even very strong SHIP-mediated inhibitory signaling [266]. Therefore, Bam32 is a SHIP-activated target of PI3K with a distinctive mechanism compared with other PH domain proteins.

The functions of Bam32 in B lymphocytes were identified gradually. A previous study revealed that Bam32 could mediate B lymphocyte proliferation *via* a mechanism involving ERK and JNK [263]. After that, Bam32 was found to induce the cytoskeletal rearrangement, the actin remodeling, the membrane ruffling, as well as the lamellipodia formation by regulating Rac-1 activation [266]. Likewise, Bam32 could increase B cell adhesion and spread on integrin ligands depending on Rac and other Rho GTPases activation [267–269]. Furthermore, Bam32 could induce B cell antigen receptor internalization. This internalization might be essential for BCR-induced JNK activation, although the in-depth mechanisms were not fully identified [270]. Besides, Bam32 deficiency resulted in the premature dissolution of the germinal center, whereas exogenous CD40 ligand could bypass this early decay [271]. Moreover, Bam32 mediated T cell-independent II antigens responses in B lymphocytes [260].

Bam32 also functions in other cell types. The absence of Bam32 led to failures not only in germline γ3 mRNA transcription but in normal antibody response to type 3 pneumonia capsular polysaccharide depended on T cell ERK activation [263,272]. The genetic evidence from LAT-knock-in (LAT-KI) mice and LAT-KI, Bam32-KO(LAT-Bam) mice supported this mechanism and added that p38 was also involved in this MAPK downstream signaling transduction [273]. Recent studies reported that Bam32 in mast cells could inhibit FcεRI-induced calcium flux and granule release by activating Lyn and SHIP negative pathways [261]. Furthermore, Bam32 in dendritic

cells (DC) located at the DC-T cell contact sites and bound to galectin-1 to suppress the proliferation of $FOXP_3^+$-induced T cells [262].

1.7 Rac and Rap—PI3K-dependent small GTPases that regulate neutrophil recruitment and ROS production

Small GTPases are a large group of signaling molecules that cycle between the GDP-bound inactive form and the GTP-bound active form. At any given time, the small GTPases remain in the inactive form, with only a small fraction becoming activated in a spatially and temporally controlled manner [274]. Only activated small GTPases can interact with downstream effectors. The intracellular molecules that balance the cycle of GTPase activation are guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs). GEFs activate the small GTPases by exchanging GDP for GTP, while GAPs, albeit termed as “activating proteins”, inactivate the small GTPases through hydrolysis [275]. Small GTPases, together with their GEFs and GAPs, regulated most if not all neutrophil functions.

The small GTPases are divided into five subfamilies: Rho, Ras, Arf, Ras-related nuclear protein (RNP), and Rab, among which Rho and Ras are the main regulators for leukocyte migration [274]. Therefore, in this section, the main focus will be on the roles of two small GTPases, Rac (a member in Rho subfamily) and Rap (a member in Ras family), and the related GEFs and GAPs in regulating neutrophil recruitment.

1.7.1 Rac and neutrophil recruitment

Rac, a subfamily of the Rho family of GTPases, has four members, Rac1, Rac2, Rac3, and RhoG. They are the most extensively studied subfamily that regulates cell movement and structural changes because they are the regulators of actin polymerization dependent on Arp2/3 in actin-rich lamellipodia [276]. Rac1, ubiquitous in various cell types, is required for the polarization and directionality in neutrophil chemotaxis. The deficiency of Rac1 in mouse neutrophils impairs the "compass" in chemotaxis *in vitro*, losing directionality while having little changes in the random

movements [277]. Similarly, the deficiency of Rac1 also suppresses neutrophil recruitment *in vivo*, both in acute inflammation of the lung and the peritoneum in mice [278]. In addition to the role of Rac1 in the formation of lamellipodia and neutrophil "compass" at the leading edge of a recruiting neutrophil, Rac1 also promotes stable polarization and tail retraction at the trailing end [276,279].

As compared to Rac1, Rac2, a small GTPase enriched in leukocytes, is predominant in regulating the migration distance and migration speed during neutrophil chemotaxis *in vitro* [277,280]. The deficiency of Rac2 largely suppresses the ability for random movements in mouse neutrophils. Although neutrophils keep the directionality to sense the gradient, they still fail to manage the efficient chemotaxis. Moreover, Rac2 is also required in the activation of neutrophilic NADPH oxidase, an enzyme for ROS production in neutrophil phagocytosis [278,280].

Given that the activation of Rac1 and Rac2 are extremely crucial in regulating neutrophil chemotaxis and recruitment, the related GEFs and GAPs are also involved in neutrophil directional movements because they balance the cycle between the active form of Rac and inactive form of Rac. GEFs P-Rex and Vav1/3 are important in maintaining the directionality neutrophil chemotaxis *in vitro* but they, together with a PI3K downstream substrate PIP3, are not involved in neutrophil recruitment *in vivo* [281–283]. GEF DOCK2, which is more related to the activation of Rac2, regulates many steps of neutrophil chemotaxis including the recruitment of Rac2 to the plasma membrane, the total migration distance, the polarization, and the directionality [284]. Moreover, the role of GEF Tiam1 in neutrophil recruitment is not discovered, whereas Tiam1 is known to be required in the polarization and chemotaxis in T lymphocytes [285]. On the contrary, Rac-related GAPs suppress neutrophil recruitment and chemotaxis because of their roles in inactivating Rac in neutrophils. In previous studies, the deficiency of GAP ArhGAP15 increased neutrophil polarization and directionality *in vitro* in Boyden chambers [286], and the deficiency of GAP ArhGAP25 enhanced acute peritonitis induced by TNFα *in vivo* [287].

It is widely believed that GEF- and GAP-mediated activation of Rac are PI3K-dependent. One of the PI3K substrates PIP3 was observed being critical in activating Rac GEFs in many previous studies [275]. The PIP3 in neutrophils activates GEFs Vav1 and P-Rex1, the latter of which promotes integrin-dependent adhesion and GPCR-dependent ROS production, respectively

[282,288]. However, knocking down the activation of Vav1/3 and P-Rex1 caused a mild loss in neutrophil chemotaxis and recruitment, suggesting that these GEFs may not be the major regulators of neutrophil migration. Knocking down the activation of PI3K-dependent GEF Tiam1 also decreased the neutrophil chemotaxis in the inflamed mouse lungs *in vivo*, although the main role of Tiam1 was discovered in the adhesion, migration, invasion, and polarity in cancer cells [289]. Moreover, the activation of atypical GEF DOCK in regulating neutrophil polarization, chemotactic speed, and persistent directionality is also dependent on the level of PIP3 [284,290]. In addition, PI3K impair host defenses and protect the host from neutrophil-inflicted damages through the activation of PI3K-dependent GAPs. The deficiency of ArhGAP15 increased neutrophil directionality and phagocytosis, whereas knocking-down of ArhGAP25 decreased neutrophil rolling velocity and increased crawling as well as transendothelial migration *in vivo* [286,287,291], suggesting that PIP3 regulates Rac-mediated neutrophil functions through the activation of Rac-related GAPs.

1.7.2 Rap and neutrophil recruitment

The roles of Rap, consisting of Rap1a, Rap1b, Rap2a, Rap2b, and Rap2c, are less revealed than those of Rac in neutrophil recruitment and activation. With the activities controlled by 10 GEFs and 10 GAPs, only the roles of Rap1 and its GEF CalDAG-GEFI have been explored in previous studies [292]. Rap1 is regarded as a key component in conducting integrin inside-out signaling [293]. The deficiency of Rap1 suppresses the F-actin polarization, further decreasing the directionality of neutrophil chemotaxis induced by LTB_4, an inflammatory modulator that triggers the expression of all β integrins in neutrophils [294]. Moreover, Rap1a, a member of Rap subfamily, facilitates E-selectin-dependent slow rolling in neutrophils [295]. Most recently, Rap1b was also revealed to suppress LPS-induced neutrophil transendothelial migration by blocking the phosphorylation of a PI3K effector Akt [296].

CHAPTER TWO

HYPOTHESES AND OBJECTIVES

2.1 Hypotheses

Bam32 was firstly discovered in human B lymphocytes from the germinal center and upregulated upon activation of BCR [257]. Sooner after, the expression of Bam32 was widely analyzed in various mouse tissues, with the top five expression levels in the lymph nodes, spleen, bone marrows, thymus, and lungs. Moreover, the expression of Bam32 in the heart, brain, liver, kidney, and skeletal muscle, is much lower albeit still detectable [263]. In immune system, Bam32 is expressed in almost all myeloid cells and lymphoid cells. ImmGen RNAseq database suggests that the expression of Bam32 in mouse neutrophils derived from the spleen, bone marrows and peritoneal cavity is even higher than B lymphocytes. This abundant expression level laid foundation for the possibility for Bam32 to function in mouse neutrophils.

Compared to the broad expression of Bam32 in different cell types, the functions of Bam32 are much less explored. Although the roles of Bam32 in the proliferation, differentiation, activation, and recruitment in adaptive immunity have been gradually established during the past twenty years, such roles in innate immunity is still very limited. A paper in 2010 revealed a suppressive role of Bam32 in mediating FcεRI-induced calcium flux and granule release in mast cells [261], suggesting for the first and the only time that Bam32 may function in innate immunity. Considering that Bam32 is significantly expressed in many innate immune cells including neutrophils as in adaptive immune cells, we hypothesize that **Bam32, as well as its upstream PI3K isoforms, regulates chemoattractant-induced neutrophil recruitment, chemotaxis, and microvascular hyperpermeability in acute inflammation.**

2.2 Objectives

Intravital microscopy combined with various molecular biological approaches are used to explore the roles of Bam32 and its upstream PI3K isoforms in neutrophil recruitment, neutrophil chemotaxis, and microvascular leakage. The specific objectives are listed as below:

2.2.1 To explore the roles of PI3K isoforms in formyl peptide-induced microvascular hyperpermeability

a) To compare the effects of deficiency of PI3K isoforms on formyl peptide-induced with chemokine-induced mouse microvascular hyperpermeability;

b) To identify the dominant cell type(s) that are crucial in the formyl peptide-induced, PI3K isoform-specific mouse microvascular hyperpermeability;

c) To elucidate the possible involvement of ROS from the dominant cell type(s) that result in the formyl peptide-induced, PI3K isoform-specific mouse microvascular hyperpermeability.

2.2.2 To explore the role of Bam32 in chemokine-induced neutrophil recruitment and chemotaxis

a) To examine the effects of deficiency of Bam32 on each step during chemokine-induced neutrophil recruitment in different mouse organs *in vivo*;

b) To examine the effects of deficiency of Bam32 on chemokine-induced mouse neutrophil chemotaxis *in vitro*;

c) To determine the effects of deficiency of Bam32 on the activation of small GTPases in chemokine-treated mouse neutrophils and reveal the possible mechanism of Bam32-dependent activation of small GTPases in chemokine-induced mouse neutrophil recruitment;

d) To determine the effects of deficiency of Bam32 on the phosphorylation of other PI3K effectors in chemokine-treated mouse neutrophils and elucidate the underlying Bam32-dependent mechanism that regulates the phosphorylation of other PI3K effectors.

2.2.3 To explore the role of Bam32 in formyl peptide-induced microvascular hyperpermeability

a) To examine the effect of deficiency of Bam32 on formyl peptide-induced mouse microvascular hyperpermeability;

b) To identify the dominant cell type(s) that are crucial in the formyl peptide-induced, Bam32-dependent mouse microvascular hyperpermeability;

c) To determine the possible role of Bam32 in neutrophil ROS generation and elucidate the possible involvement of neutrophil ROS in formyl peptide-induced, Bam32-dependent mouse microvascular hyperpermeability;

d) To reveal the possible involvement of MAPK signaling pathways in formyl peptide-induced, Bam32-dependent ROS generation and mouse microvascular hyperpermeability.

CHAPTER THREE

CRITICAL ROLE FOR PI3Kγ-DEPENDENT NEUTROPHIL REACTIVE OXYGEN SPECIES IN WKYMVm-INDUCED MICROVASCULAR HYPERPERMEABILITY

3.1 Preface

In this chapter, we reveal a pivotal role for neutrophil PI3Kγ, which is an upstream regulator of Bam32, in WKYMVm-induced microvascular hyperpermeability through modulating the production of ROS in recruiting neutrophils. This study shows the importance of neutrophil PI3Kγ-specific signaling pathway in regulating microvasculature in acute inflammation when formyl peptides are involved.

This chapter has been published as a research paper by Li Hao, Xi Lei, Hong Zhou, Aaron J. Marshall, and Lixin Liu in the *Journal of Leukocyte Biology* 2019 June 19. Contents of this chapter have been adapted from the published article with the permission from the *Journal of Leukocyte Biology*. In this study, Li Hao conducted all the experiments, performed data analysis, drafted the manuscript, and participated in the study design, data interpretation and manuscript revision.

3.2 Abstract

PI3K has been implicated in regulating microvascular permeability changes during inflammation. However, its role in neutrophil-driven microvascular leakage in acute inflammation remains unclear. Using intravital microscopy in mice, we examined the role of PI3Kγ and PI3Kδ in formyl peptide WKYMVm- and chemokine CXCL2-induced permeability changes and assessed simultaneously neutrophil adhesion and emigration in post-capillary venules of the murine cremaster muscle. We found a PI3Kγ-specific mechanism in WKYMVm-induced but not CXCL2-induced microvascular hyperpermeability. The increased microvascular permeability triggered by WKYMVm was not entirely due to neutrophil adhesion and emigration in cremasteric microvasculature in different PI3K transgenic mouse strains. The PI3Kγ-specific hyperpermeability was neutrophil-mediated as this was reduced after the depletion of neutrophils in mouse circulation. Chimeric mice with PI3Kγ-deficient neutrophils but wild-type endothelium also showed reduced hyperpermeability. Furthermore, we found that the catalytic function of PI3Kγ was required for reactive oxygen species (ROS) generation in neutrophils stimulated with WKYMVm. Pharmacological scavenging PI3Kγ-dependent ROS in the tissue eliminated the discrepancy in hyperpermeability between different PI3K transgenic mice and alleviated WKYMVm-induced microvascular leakage in all mouse strains tested. In conclusion, our study uncovers the critical role for PI3Kγ-dependent ROS generation by neutrophils in formyl peptide-induced microvascular hyperpermeability during neutrophil recruitment.

3.3 Introduction

PI3K is a family of structurally and functionally related intracellular kinases regulating many cellular functions and several key events in tissues including microvascular leakage. The class I PI3K isoforms (α, β, γ, and δ) are important regulators of vascular integrity and contribute independently to the progression of inflammation in the endothelium. The PI3Kγ and PI3Kβ isoforms can be activated by G-protein-coupled receptors, while PI3Kα, PI3Kβ, and PI3Kδ are activated by tyrosine kinase receptors after extracellular ligand-binding [218]. Previous studies revealed that PI3K-mediated signaling in mast cells can regulate vascular leakage in asthma [297]. The histamine released from mast cells resulted in the disruption of endothelial cell function *via* the PI3K/Akt signaling pathway [298,299]. PI3K signaling was also involved in sphingosine-1-phosphate receptor-2 (S1PR2)-mediated paracellular permeability increases by opening adherens junctions between the neighboring endothelial cells [137]. Furthermore, the increased activity of PI3K/Akt signaling was observed in PDGF-mediated endothelial barrier impairment caused by cocaine exposure, although the role of specific involving PI3K isoforms remains unclear [300].

Several studies revealed the role of specific PI3K isoforms in vascular hyperpermeability during acute inflammation. First, PI3Kα regulated Pyk2- and Rac1-mediated endothelial functions *via* inducing the formation of a VE-cadherin-associated protein complex during vascular leakage [301]. Second, PI3Kδ was required in VEGF signaling in increasing microvascular permeability, and blocking PI3Kδ or PI3Kγ attenuated microvascular hyperpermeability but not angiogenesis [302,303]. Third, PI3Kγ played an important role in the CXCR2/Rac1 signaling axis that was required in IL-8-induced vascular leakage [110]. Besides the functional role downstream of CXCR2 signaling, the activation of PI3Kγ protected the lung from septic vascular injury and edema formation in the mouse *E. coli* sepsis model but increased the permeability of blood-brain barrier by suppressing intracellular cAMP level in LPS-treated microglial cells [304,305]. The distinct role of different PI3K isoforms in microvascular permeability during acute inflammation, however, remains ill-defined.

Trp-Lys-Tyr-Met-Val-D-Met-NH2 (WKYMVm) is a synthetic peptide that mimics bacterial formyl peptide f-Met-Leu-Phe (fMLP) and activates neutrophils via binding the formyl peptide receptors (FPR) [306,307]. Beyond a peptide that triggers immune responses, WKYMVm and its analogs also play a role in vascular dysfunction. WKYMVm reverses vascular hyporeactivity to phenylephrine induced by LPS in mouse aorta through decreasing the production of nitric oxide [308]. LL-37, another FPR agonist, was reported to change smooth muscle contractility *via* increasing the levels of endothelium-dependent factors [309]. As all these previous studies are limited to investigating the functions of FPR agonists on large vessels, the WKYMVm-induced alterations in microvasculature remain unknown.

In the present study, we utilize intravital microscopy and fluorescence imaging *in vivo* to investigate the PI3K isoform-specific mechanisms present in WKYMVm-induced but not in chemokine CXCL2-induced permeability increases in post-capillary venules. We explore the role of PI3Kγ-dependent reactive oxygen species (ROS) generation by neutrophils in the impairment of microvascular barrier functions during neutrophil recruitment.

3.4 Material and methods

3.4.1 Animal

PI3Kγ-deficient (PI3Kγ$^{-/-}$) mice were generated by Dr. J.M. Penninger [310]. PI3Kδ-mutant (PI3Kδ$^{D910A/D910A}$) mice were made by Dr. B. Vanhaesebroeck [311]. Both mouse strains were on the C57BL/6 background and transferred to the University of Saskatchewan. Male mice used in experiments were between 6 and 12 weeks of age along with age-matched male C57BL/6N mice (wild-type, WT) purchased from Charles River Canada (Saint-Constant, QC, Canada). This study was carried out with the approval of the University Committee on Animal Care and Supply at the University of Saskatchewan and following the standards of the Canadian Council on Animal Care. All efforts were made to reduce animal suffering and all surgeries were performed under deep ketamine-xylazine anesthesia.

3.4.2 Measurement of microvascular permeability and neutrophil adhesion and emigration

Mice were anesthetized after an intraperitoneal (i.p.) injection with a cocktail of ketamine (200 mg/kg, Roger, Montreal, QC, Canada) and xylazine (10 mg/kg, Bayer, Toronto, ON, Canada). Jugular vein cannulation was performed to allow subsequent administration of FITC-labeled BSA (Sigma–Aldrich, Oakville, ON, Canada). The mouse cremaster muscle preparation was used as described previously [312,313]. The exposed cremaster muscle was superfused with 37°C-warmed bicarbonate-buffered physiological saline (pH 7.4; containing in mM, NaCl 133.9, KCl 4.7, MgSO4 1.2, and NaHCO3 20.0; all reagents purchased from Fisher Scientific, Toronto, ON, Canada). An upright microscope (BX61WI; Olympus, Tokyo, Japan) with a LUCPLFLN 20× objective lens was used for bright-field and fluorescent intravital microscopy. The permeability index, defined as the ratio of extravascular fluorescence intensity to the adjacent endovascular fluorescence intensity of the observed cremasteric venule, was measured as described [314–316]. Briefly, FITC-labeled BSA at 25 mg/kg was infused into the mice through the jugular vein 5 min prior to 1 h superfusion of the exposed cremaster muscle with WKYMVm (0.1 μM, Phoenix Pharmaceutical, Burlingame, CA) [317] or murine CXCL2 (0.5 nM, R&D Systems, Minneapolis,

MN) [38] or the control saline. Fluorescence intensity (excitation wavelength: 495 nm; emission wavelength: 525 nm) was detected using a monochrome deep-cooled CCD camera (Retiga SRV, QImaging, Surrey, BC, Canada) and quantified by the MAG Biosystems software (MetaMorph®, Molecular Devices Inc., PA). Fluorescence photographs were taken on the cremasteric venule every 5 min during superfusion with WKYMVm, CXCL2, or the saline. The numbers of adhered and emigrated neutrophils were determined under bright-field microscopy prior to (0 min) and after 60-min treatment with WKYMVm, CXCL2, or the saline as described previously [312]. The emigrated neutrophils were defined as the neutrophils emigrated to the extravascular tissue from the venule, excluding the neutrophils that attached on the tunica media. The number of emigrated neutrophils in intravital microscopy was counted in the field of 443×286 μm^2 (two full screens on the monitor) adjacent to the recorded venule. All of the above reagents were prepared in 37°C-warmed bicarbonate-buffered saline before superfusion.

3.4.3 Neutrophil depletion in vivo

Anti-mouse Ly-6G (Gr-1) functional-grade purified Abs (200 μg, i.p. with saline, clone RB6-8C5, eBioscience, Santa Clara, CA) were administrated to mice 24 h prior to intravital microscopy and permeability index determination. The number of neutrophils in the circulation was <2% after 24 h anti-Ly-6G treatment [318]. The leukocyte rolling flux (the number of leukocytes passed through a venular cross-section in 1 min) was determined to verify neutrophil depletion during brightfield intravital microscopy.

3.4.4 Harvest of neutrophils from mouse bone marrow

Neutrophils were freshly isolated from the bone marrow of WT, PI3K$\delta^{D910A/D910A}$, and PI3K$\gamma^{-/-}$ mice. The femur and tibia were dissected immediately after each mouse was sacrificed, and the marrow cavity was flushed with ice-cold calcium- and magnesium-free PBS. The bone marrow cells enriched in flushing fluid were separated by 3-density (72%, 64%, and 52%) Percoll (GE Healthcare, Baie d'Urfe, QC, Canada) gradient centrifugation at 1060 × g for 30 min as

described previously [319]. This method yielded over 85% morphologically mature neutrophils [320].

3.4.5 Bone marrow transplantation

To explore the functions of different PI3K isoforms in neutrophils on the same WT endothelial background, we generated three different types of chimeric mice (designated as WT→WT, PI3K$\delta^{D910A/D910A}$→WT, and PI3K$\gamma^{-/-}$→WT) by transplantation of bone marrow from 6 week-old WT, PI3K$\delta^{D910A/D910A}$, or PI3K$\gamma^{-/-}$ mice into 6-week-old WT recipient mice. Before bone marrow cell injection, all recipients received 2 doses of X-ray irradiation (500 cGy/dose), with a 3-h interval. The freshly prepared bone marrow cells ($6-8 \times 10^6$ cells) from each of the 3 strains of donors were injected through the tail veins into WT recipient mice, respectively. Thereafter enrofloxacin (200 mg/L) freshly prepared in sterile drinking water was provided to the chimeric mice over the following 2 weeks, along with sterile food. The mice were housed in sterile cages for 6 weeks after the bone marrow transplantation to reconstitute full humoral immunity before use.

3.4.6 Determination of extracellular hydrogen peroxide generation

Bone marrow-derived mouse neutrophils were freshly prepared as described above and resuspended in 37°C-warmed Krebs-Ringer phosphate glucose solution (containing in mM, NaCl 145, Na_2HPO_4 5.7, KCl 4.86, $CaCl_2$ 0.54, $MgSO_4$ 1.22 and glucose 5.5, pH 7.35). An Amplex Red Hydrogen Peroxide assay kit (Thermo Fisher) was used to detect the production of extracellular hydrogen peroxide in neutrophils with blank Krebs-Ringer phosphate glucose buffer, specific PI3Kγ inhibitor CZC24832 (10 μM, Cayman Chemical, Ann Arbor, MI) [321], NADPH oxidase inhibitor diphenyleneiodonium (DPI, 40 μM, Sigma-Aldrich) [322] or superoxide dismutase (SOD) mimetic MnTBAP (200 μM, Santa Cruz Biotechnology, Dallas, TX) [323], respectively. Briefly, after 30 min incubation with the blank buffer or inhibitors, 20 μL of 1.0×10^6/mL neutrophils were mixed with the reaction mixture which contained 50 μM Amplex Red reagent and 0.1 U/mL HRP in 100 μL Krebs-Ringer phosphate glucose buffer in a black 96-well microplate. The negative

control was prepared by adding 20 μL of Krebs-Ringer Phosphate glucose buffer without cells to a separate 100 μL warmed reaction mixture. Fluorescent signals (excitation: 578 nm and emission: 590 nm) were captured by Fluoroskan Ascent Microplate Fluorometer (Thermo Fisher) upon and every 10 min after stimulation of WKYMVm (0.1 μM). Over the 1-hour time period, the microplate was returned to the 37°C incubator after every measurement for keeping reaction temperature. The relative fluorescent signals represented the values of dividing the original fluorescent signal in each well by the fluorescent signal of the negative control at the corresponding time point.

3.4.7 Inhibition of ROS generation in vivo

Mice were prepared for intravital microscopy and post-capillary venule permeability was measured in the cremaster muscle as described above. The DPI and MnTBAP were freshly prepared separately in 37°C-warmed bicarbonate-buffered saline. The cremaster muscle was superfused with DPI (40 μM) or MnTBAP (200 μM) for 30 min prior to the addition of WKYMVm or CXCL2. Fluorescence photographs were taken every 5 min after WKYMVm was applied and permeability indices were determined at each time point as described above.

3.4.8 Statistical analysis

Data are expressed as arithmetic means ± SEM from at least three independent experiments. Statistical analyses of the differences among groups were performed using Student *t*-test or one-way ANOVA followed by the Holm-Sidak method. The *t*-test was two-tailed, with $p<0.05$ considered to be statistically significant. Analyses were conducted using Statistical Product and Service Solution (SPSS) version 13.0 (SPSS, Chicago, IL) and GraphPad Prism 7 (GraphPad Software, La Jolla, CA).

3.5 Results

3.5.1 Time courses for WKYMVm- and CXCL2-induced microvascular hyperpermeability, neutrophil adhesion and emigration

The dose of FPR agonist WKYMVm for this study was chosen based on the previous study [324], in which 0.1 μM WKYMVm induced maximally ROS production in human monocytes. To verify that 0.1 μM WKYMVm is a suitable dose, we determined the permeability index changes, neutrophil adhesion, and neutrophil emigration over time in WT mouse cremaster muscle by using four different doses of WKYMVm with 10-fold in each gradient. As shown in Figure 3-1, 0.1 μM and 1 μM WKYMVm induced similar elevation pattern in hyperpermeability, neutrophil adhesion and emigration. In contrast, 0.01 μM WKYMVm induced significantly lower levels of hyperpermeability and neutrophil emigration at 60 min. Moreover, 10 μM WKYMVm induced slightly but not significantly higher levels of hyperpermeability and neutrophil adhesion as compared to the responses induced by 0.1 μM and 1 μM WKYMVm. Considering that the ideal dose for this study should preferably induce a significant increase in hyperpermeability, neutrophil adhesion, and neutrophil emigration while avoiding being sufficient to induce systematic inflammatory responses, we picked 0.1 μM as the dose of WKYMVm throughout this study.

In like manner, the dose of CXC chemokine CXCL2 for this study was chosen based on the previous studies as well as the time courses for four different doses of CXCL2 with 10-fold in each gradient. In the previous study, the dose of 0.5 nM and 5 nM CXCL2 both significantly induced neutrophil adhesion and emigration. As depicted in Figure 3-2, 0.5 nM and 5 nM CXCL2 induced similar levels of hyperpermeability and neutrophil adhesion, whereas 5 nM CXCL2 induced significantly higher neutrophil emigration. In contrast, 0.05 nM CXCL2 failed to induce hyperpermeability, while 50 nM CXCL2 induced similar levels of hyperpermeability, neutrophil adhesion, and neutrophil emigration as compared to 5 nM CXCL2. Throughout our study, the lower but suboptimal dose of CXCL2 (0.5 nM) was chosen to avoid counting overly crowded adherent neutrophils in the venule and for better quantification of transmigrated neutrophils.

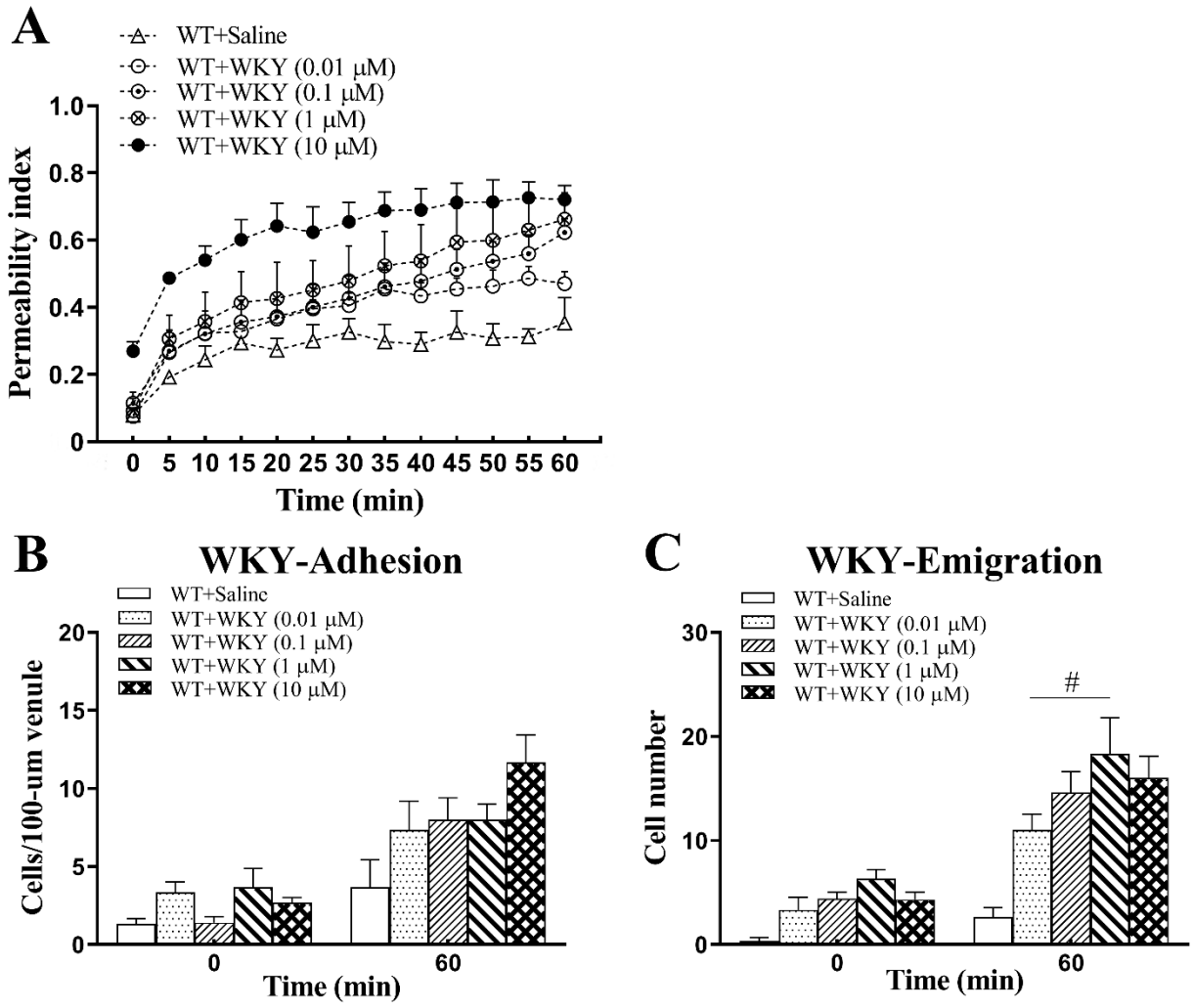

Figure 3-1. Dose-dependent responses of WKYMVm on microvascular hyperpermeability and neutrophil recruitment in wild-type mice. Permeability index of mouse cremasteric post-capillary venules was measured following 1-h superfusion of bicarbonate-buffered saline or various doses of WKYMVm (WKY, 0.01–10 μM, A). Adhesion cell number of 100-μm length of venule (B) and emigration cell number (cells/443×286 μm^2 field, C) were determined simultaneously in the same venule of bicarbonate-buffered saline-treated or WKYMVm-induced cremasteric microvascular hyperpermeability. A–C, mean ± SEM of 3 mice per group. # indicates significant differences ($p<0.05$) between WKYMVm doses of 0.01 μM and 1 μM.

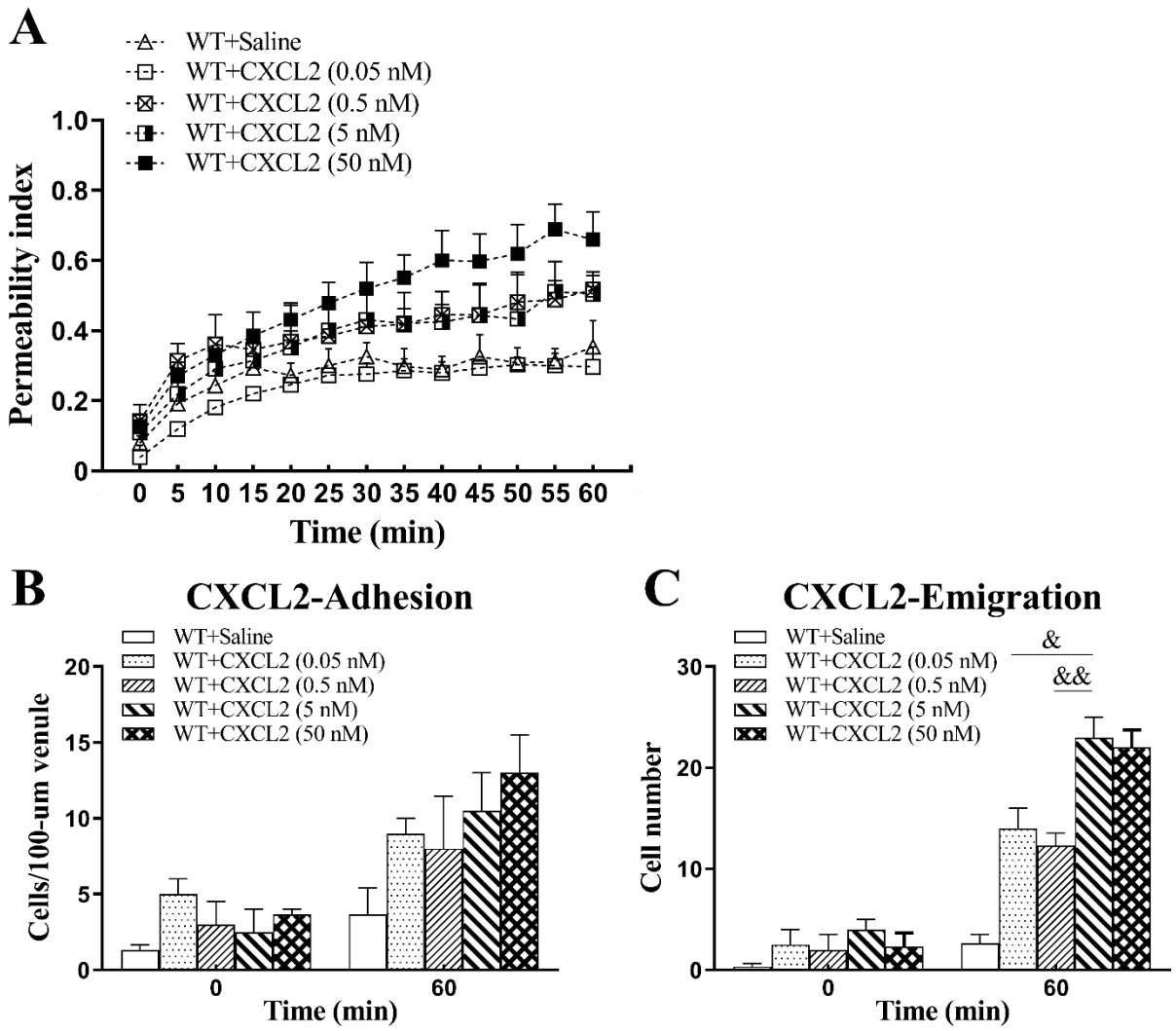

Figure 3-2. Dose-dependent responses of CXCL2 on microvascular hyperpermeability and neutrophil recruitment in wild-type mice. Permeability index of mouse cremasteric post-capillary venules was measured following 1-h superfusion of bicarbonate-buffered saline or various doses of CXCL2 (0.05–50 nM, A). Adhesion cell number of 100-μm length of venule (B) and emigration cell number (cells/443×286 μm^2 field, C) were determined simultaneously in the same venule of bicarbonate-buffered saline-treated or CXCL2-induced cremasteric microvascular hyperpermeability. A–C, mean ± SEM of 3 mice per group. & and && indicate significant differences ($p<0.05$ and $p<0.01$, respectively) between CXCL2 doses of 0.05 nM and 5 nM and between 0.5 nM and 5 nM, respectively.

3.5.2 WKYMVm-induced microvascular hyperpermeability, neutrophil adhesion and emigration in WT, PI3Kδ$^{D910A/D910A}$ mice and PI3Kγ$^{-/-}$ mice

To determine the impact of PI3Kγ and PI3Kδ inactivation on murine post-capillary venule barrier functions and neutrophil recruitment, we utilized intravital microscopy analyses of the cremaster muscle. We first determined the permeability index change induced by WKYMVm, a parameter representing the degree of vascular leakage from the cremasteric venule. The baseline vascular permeability alteration upon saline superfusion in fluorescence intravital microscopy did not differ significantly between WT, PI3Kγ$^{-/-}$ and PI3Kδ$^{D910A/D910A}$ mice. After superfusion with 0.1 μM WKYMVm, microvascular permeability in cremasteric venules substantially increased in both WT and PI3Kδ$^{D910A/D910A}$ mice, but the time-dependent elevation was significantly less in PI3Kγ$^{-/-}$ mice in as early as 5 min and all the time points thereafter during 1-h WKYMVm superfusion (Figure 3-3A). Because WKYMVm not only increases the venular permeability but also triggers neutrophil extravasation as an end-target chemoattractant [36], we simultaneously determined the number of adherent and emigrated neutrophils in all three mouse strains before and after 60-min WKYMVm superfusion. Compared to WT mice, PI3Kδ-mutant mice had a significantly reduced number of adherent neutrophils and subtly but insignificantly reduced emigrated neutrophils after 60 min superfusion with WKYMVm (Figure 3-3B and 3-3C). Moreover, PI3Kγ$^{-/-}$ mice had substantially reduced numbers of adherent neutrophils and emigrated neutrophils compared to WT mice. However, PI3Kγ$^{-/-}$ mice only had significantly reduced numbers of adherent neutrophils but not emigrated neutrophils compared to PI3Kδ-mutant mice (Figure 3-3B and 3-3C). These results reveal that, during WKYMVm-induced neutrophil recruitment, although both PI3Kδ and PI3Kγ have an important role in neutrophil adhesion and emigration, only PI3Kγ is essential in mediating microvascular permeability increases.

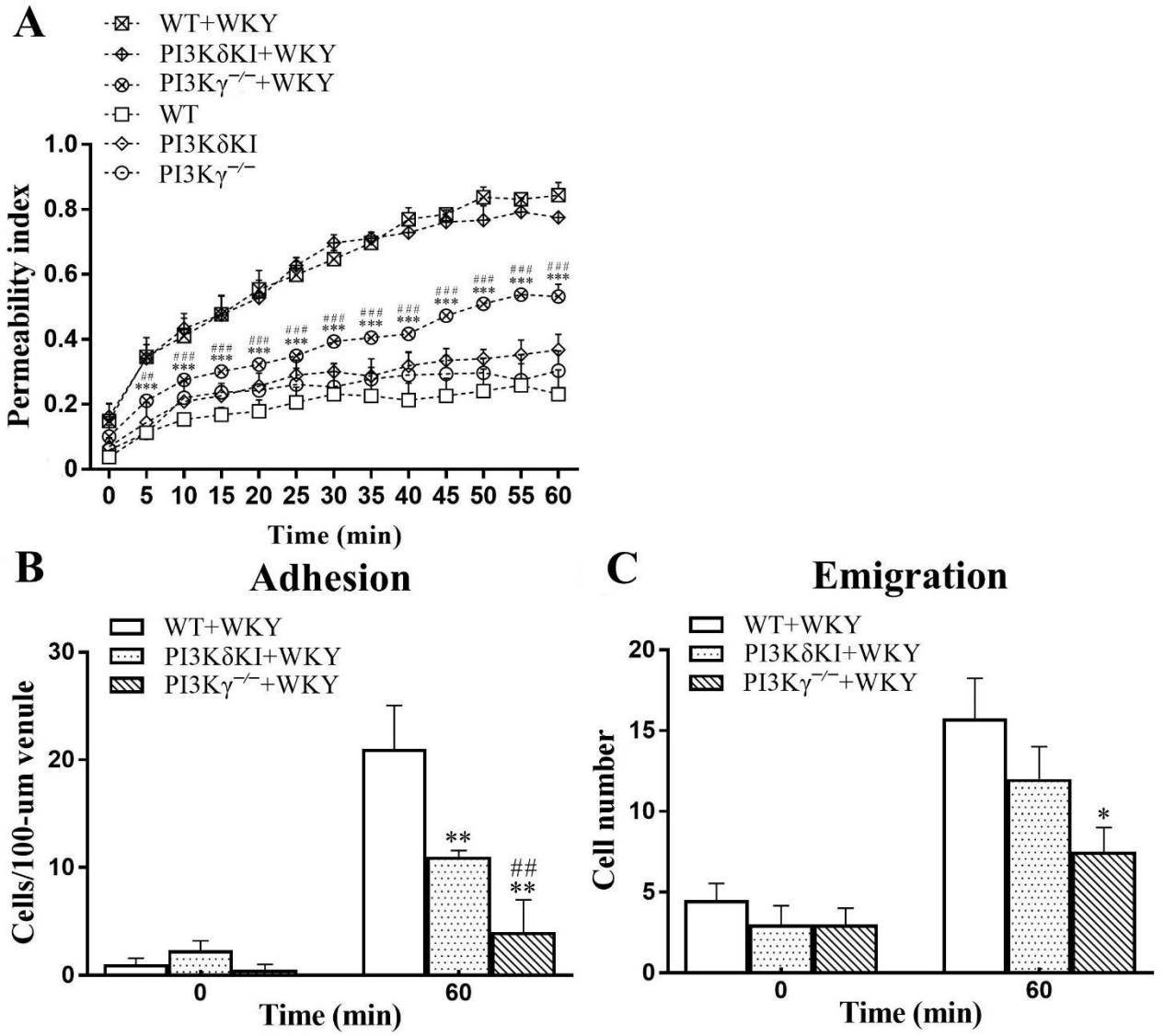

*Figure 3-3. WKYMVm-induced microvascular hyperpermeability and neutrophil recruitment in wild-type, PI3K$\delta^{D910A/D910A}$ and PI3K$\gamma^{-/-}$ mice. (A) Permeability indices of mouse cremasteric post-capillary venules was measured following 1-h superfusion of bicarbonate-buffered saline or WKYMVm (WKY, 0.1 μM). Adhesion cell number of 100-μm venule (B) and emigration cell number (cells/443×286 μm^2 field, C) were determined simultaneously in the same experiment of WKYMVm-induced (0.1 μM) cremasteric microvascular hyperpermeability before (0 min) and 60 min following WKYMVm superfusion. A–C, mean ± SEM of 4 mice per group. *, ** and *** indicate significant differences ($p<0.05$, $p<0.01$, and $p<0.001$, respectively) from wild-type (WT) mice. ## and ### indicate significant differences ($p<0.01$ and $p<0.001$, respectively) from PI3K$\delta^{D910A/D910A}$ (PI3KδKI) mice.*

3.5.3 CXCL2-induced microvascular hyperpermeability, neutrophil adhesion and emigration in WT, PI3Kδ$^{D910A/D910A}$ mice and PI3Kγ$^{-/-}$ mice

To investigate whether this PI3Kγ isoform-specific function is also required in responses to other neutrophil chemoattractants, we substituted WKYMVm for chemokine CXCL2, which is a strong intermediary neutrophil chemoattractant. Interestingly, superfusion with 0.5 nM CXCL2 increased the permeability indices, although to a lesser extent than WKYMVm, but no PI3K isoform-specific discrepancy is observed under this condition (Figure 3-4A). Non-functional PI3Kδ-mutant had a lower but insignificant decrease in the numbers of adherent and emigrated neutrophils after 60 min superfusion with CXCL2, whereas deficiency of PI3Kγ significantly reduced the numbers of both adherent and emigrated cells (Figure 3-4B and 3-4C). Together, our results indicate an important role for PI3Kγ in neutrophil recruitment in both models, and the WKYMVm model reveals an additional role for PI3Kγ in WKYMVm- but not CXCL2-induced microvascular hyperpermeability.

3.5.4 Neutrophil depletion attenuated the PI3K isoform-specific differences in WKYMVm-induced microvascular hyperpermeability

Both neutrophils and endothelial cells contribute to the deterioration of microvascular leakage stimulated by chemoattractants such as WKYMVm. To determine the role of neutrophils in regulating microvascular hyperpermeability in our mouse model, we examined the permeability changes of post-capillary venules after depleting neutrophils in the circulation using anti-mouse Ly-6G antibodies. Intraperitoneal injection of these antibodies for 24 h depleted over 98% neutrophils in the mouse circulation [318]. In neutrophil-depleted WT mice, the microvascular leakage induced by WKYMVm was significantly decreased and occurred at a much slower rate compared to WT mice without depletion of neutrophils (Figure 3-5 and 3-6). Moreover, neutrophil depletion eliminated the differences in microvascular hyperpermeability among WT, PI3Kγ$^{-/-}$ and PI3Kδ$^{D910A/D910A}$ mouse strains (Figure 3-5 and 3-6). These results indicate that the inflammatory role of PI3Kγ in WKYMVm-triggered microvascular hyperpermeability is derived from the PI3Kγ in recruiting neutrophils in the microvasculature.

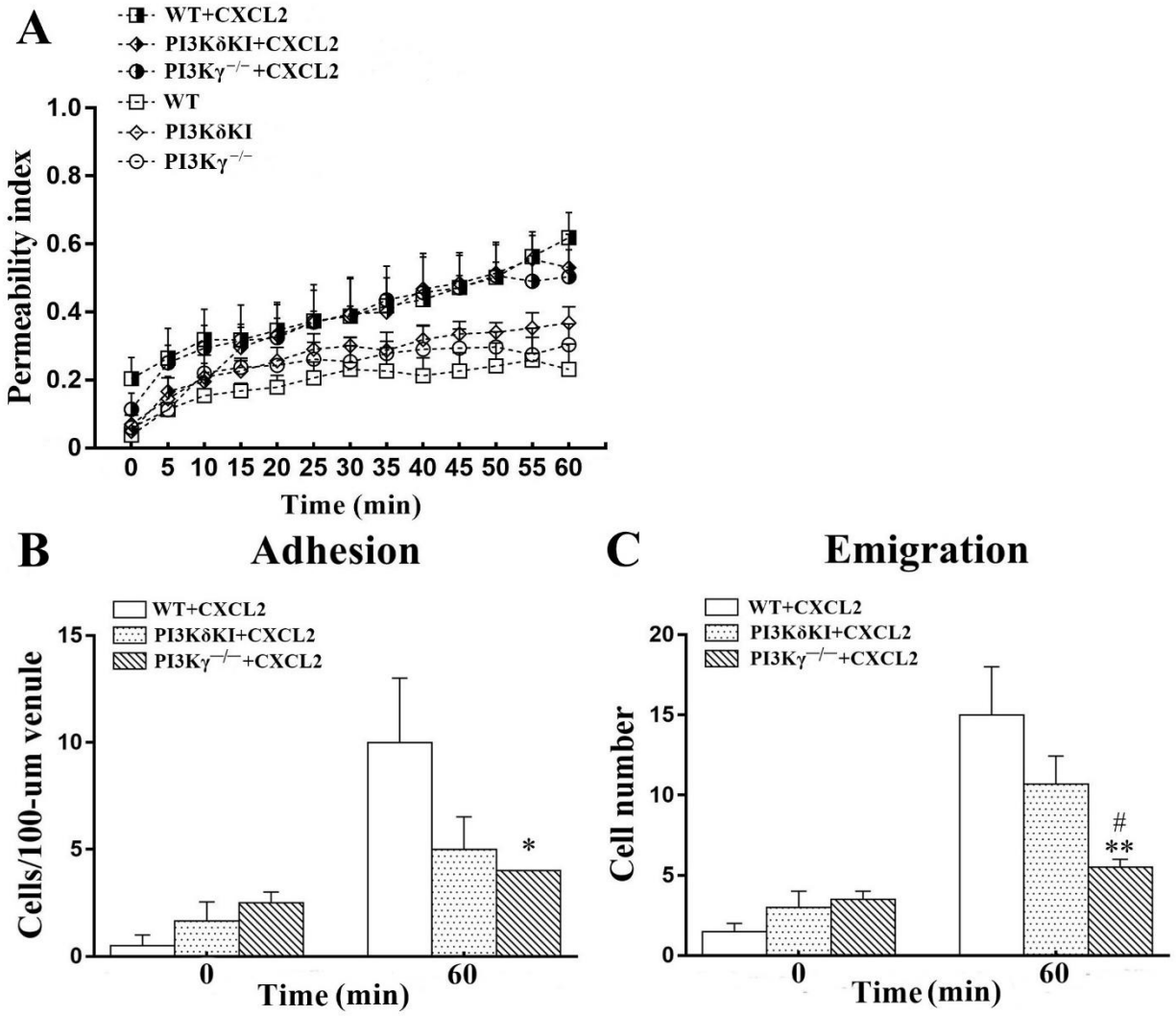

*Figure 3-4. CXCL2-induced microvascular hyperpermeability and neutrophil recruitment in wild-type, PI3K$\delta^{D910A/D910A}$ and PI3K$\gamma^{-/-}$ mice. Permeability indices of mouse cremasteric post-capillary venules was measured following 1-h superfusion of bicarbonate-buffered saline or CXCL2 (0.5 nM, A). Adhesion cell number of 100-μm venule (B) and emigration cell number (cells/443×286 μm^2 field, C) were determined simultaneously in the same experiment of CXCL2-induced (0.5 nM) cremasteric microvascular hyperpermeability before (0 min) and 60 min following CXCL2 superfusion. A–C, mean ± SEM of 4 mice per group. * and ** indicate significant differences ($p<0.05$ and $p<0.01$, respectively) from wild-type (WT) mice. # indicates a significant difference ($p<0.05$) from PI3K$\delta^{D910A/D910A}$ (PI3KδKI) mice.*

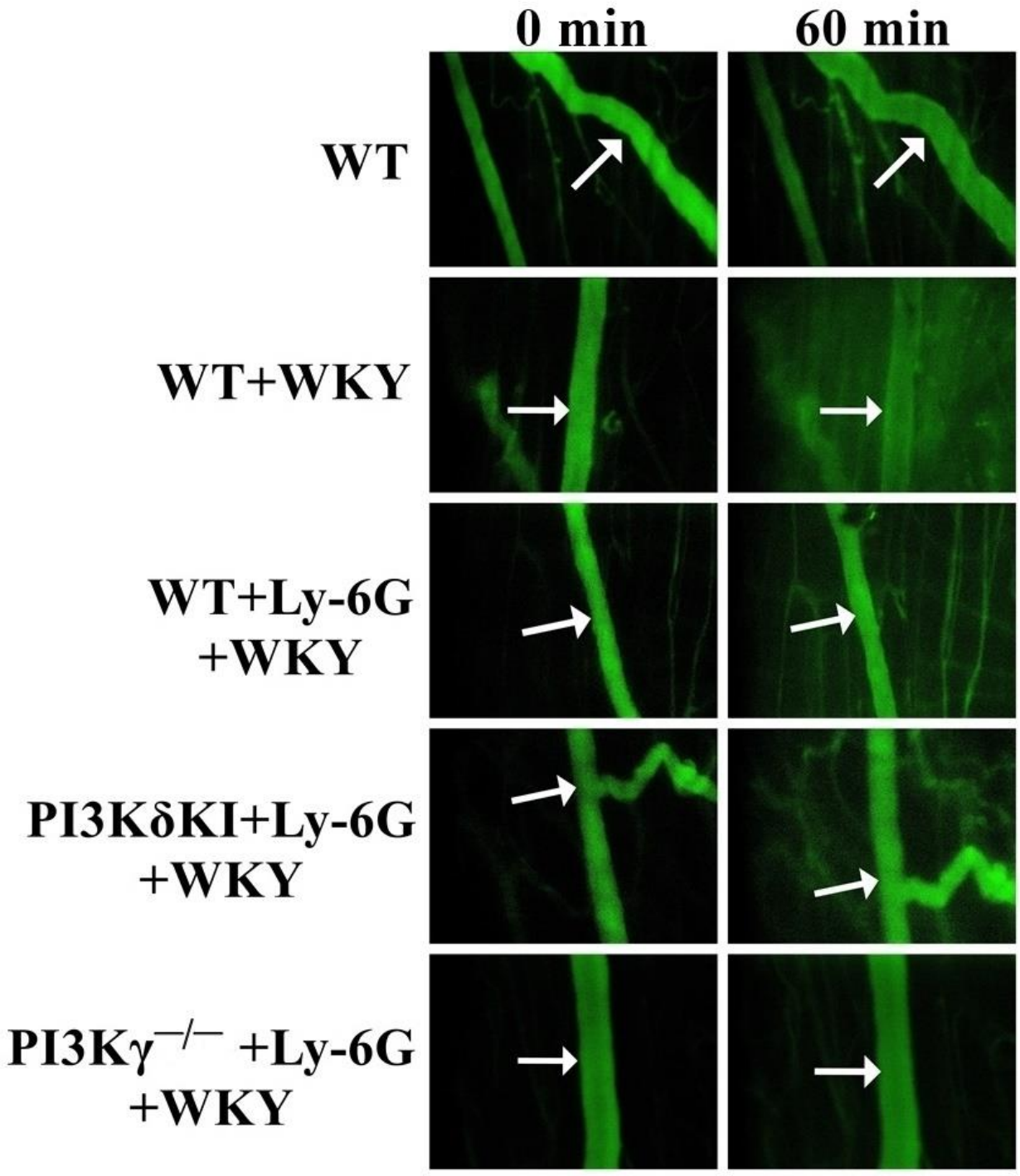

Figure 3-5. Effect of neutrophil depletion on WKYMVm-induced microvascular hyperpermeability in wild-type, PI3K$\delta^{D910A/D910A}$ and PI3K$\gamma^{-/-}$ mice (micrographs). Representative fluorescence intravital micrographs of mouse cremasteric post-capillary venules were taken before (0 min) and 60 min after injection of FITC-labelled BSA (25 mg/kg, i.v.) in wild-type (WT) mice, PI3K$\delta^{D910A/D910A}$ (PI3KδKI) mice, and PI3K$\gamma^{-/-}$ mice. Arrow indicates the venular segment observed for permeability index measurements (Magnification: ×20).

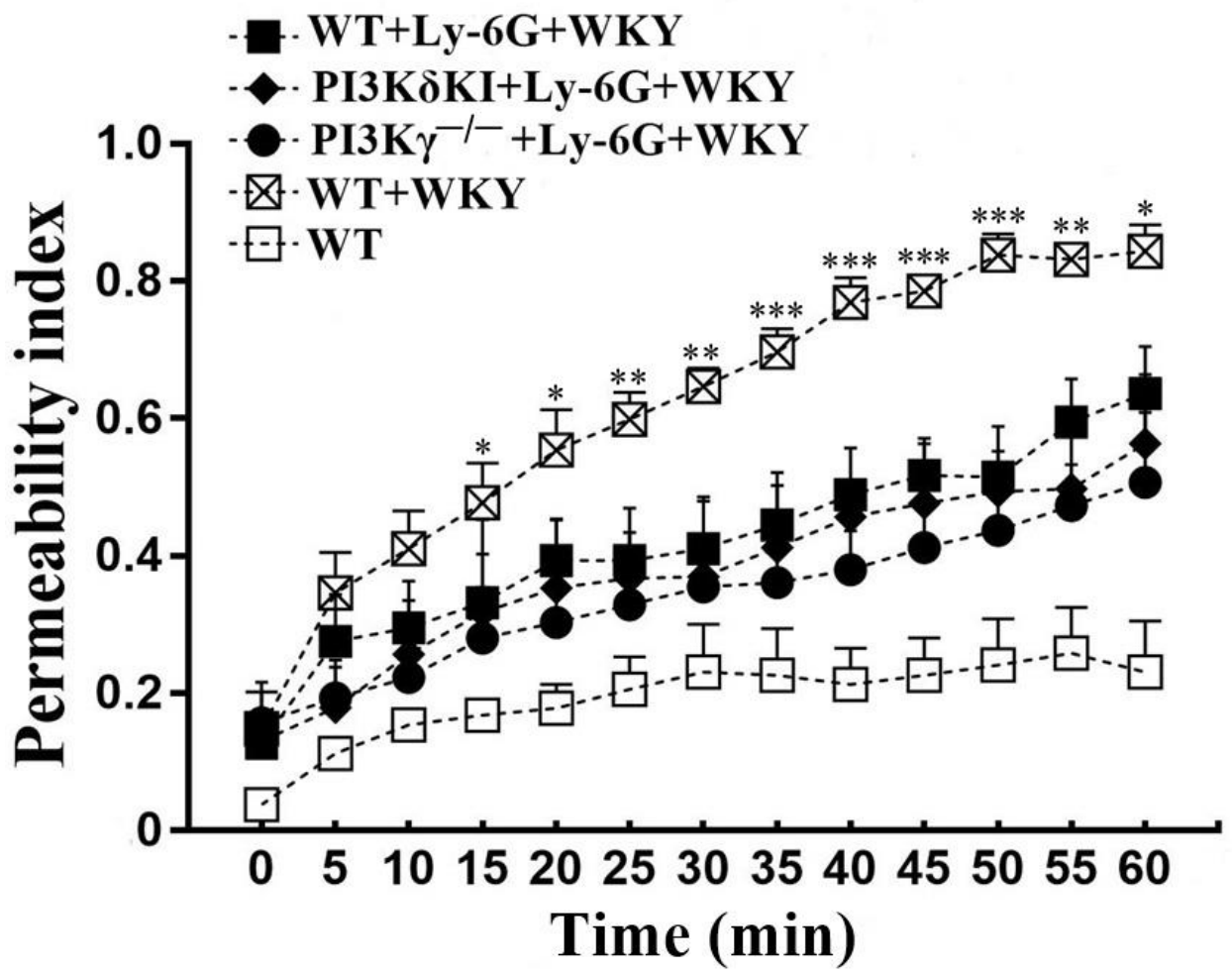

*Figure 3-6. Effect of neutrophil depletion on WKYMVm-induced microvascular hyperpermeability in wild-type, PI3K$\delta^{D910A/D910A}$ and PI3K$\gamma^{-/-}$ mice (quantification results). Permeability indices of mouse cremasteric post-capillary venules was measured following 1-h bicarbonate-buffered saline superfusion (white square) or 1-h WKYMVm (WKY, 0.1 μM) superfusion in the mice with (black symbols) or without (white square with cross) Ly-6G antibody (200 μg, i.p.) 24-h treatment prior to WKYMVm superfusion during experiment of intravital microscopy. Mean ± SEM of 4 mice per group. *, **, and *** indicate significant differences between groups of WT+WKY and WT+Ly-6G+WKY ($p<0.05$, $p<0.01$ and $p<0.001$, respectively).*

3.5.5 WKYMVm-induced microvascular hyperpermeability, neutrophil adhesion, and neutrophil emigration in three types of chimeric mice

To verify the role of PI3Kγ in the recruiting neutrophils, we generated three types of bone marrow chimeric mice (WT→WT, PI3K$\delta^{D910A/D910A}$→WT and PI3K$\gamma^{-/-}$→WT) which shared the same WT endothelial background but had different genotypes of neutrophils (WT, PI3K$\delta^{D910A/D910A}$ and PI3K$\gamma^{-/-}$, respectively) in the circulation. Without WKYMVm superfusion, no significant difference in vascular permeability increases was observed among three types of chimeric mice. However, superfusion of WKYMVm induced higher microvascular leakage in WT→WT and PI3K$\delta^{D910A/D910A}$→WT mice than in PI3K$\gamma^{-/-}$→WT mice (Figure 3-7 and 3-8A). This difference was significant starting from 35 min and 45 min when PI3K$\gamma^{-/-}$→WT mice were compared with WT→WT and PI3K$\delta^{D910A/D910A}$→WT mice, respectively. The numbers of adherent and emigrated cells after 60 min superfusion of WKYMVm were significantly reduced in PI3K$\gamma^{-/-}$→WT mice compared to WT→WT mice, whereas the significant reductions were not observed in PI3K$\delta^{D910A/D910A}$→WT mice (Figure 3-8B and 3-8C). These results further confirm the inflammatory role of PI3Kγ in neutrophils in promoting mouse microvascular hyperpermeability that is sensitive to WKYMVm.

3.5.6 Time courses for WKYMVm- and CXCL2-induced generation of extracellular ROS in bone marrow-derived neutrophils

Among many neutrophil-released factors that contribute to acute changes of permeability in post-capillary venules, the function of PI3Kγ-dependent ROS needs to be addressed. First of all, the generation of extracellular ROS induced by different doses of WKYMVm and CXCL2 were determined in isolated WT neutrophils. Among the three doses shown in Figure 3-9A, 0.01 μM WKYMVm induced the minimal generation of hydrogen peroxide, whereas 1 μM induced the most significant generation of hydrogen peroxide at 10 min and 20 min. However, the chosen dose for the following study on ROS generation was 0.1 μM which was suboptimal because of the possibility that the lower concentration of WKYMVm (0.1 μM) used *in vivo* was below the threshold for inducing the differences in neutrophilic ROS generation among three mouse strains needed to be excluded.

Interestingly, a 100-fold increase of CXCL2 to final concentration 50 nM still failed to induce the generation of extracellular hydrogen peroxide (Figure 3-9B). The results indicate that CXCL2, unlike WKYMVm, may increase microvascular permeability through other mechanisms rather than through inducing ROS production.

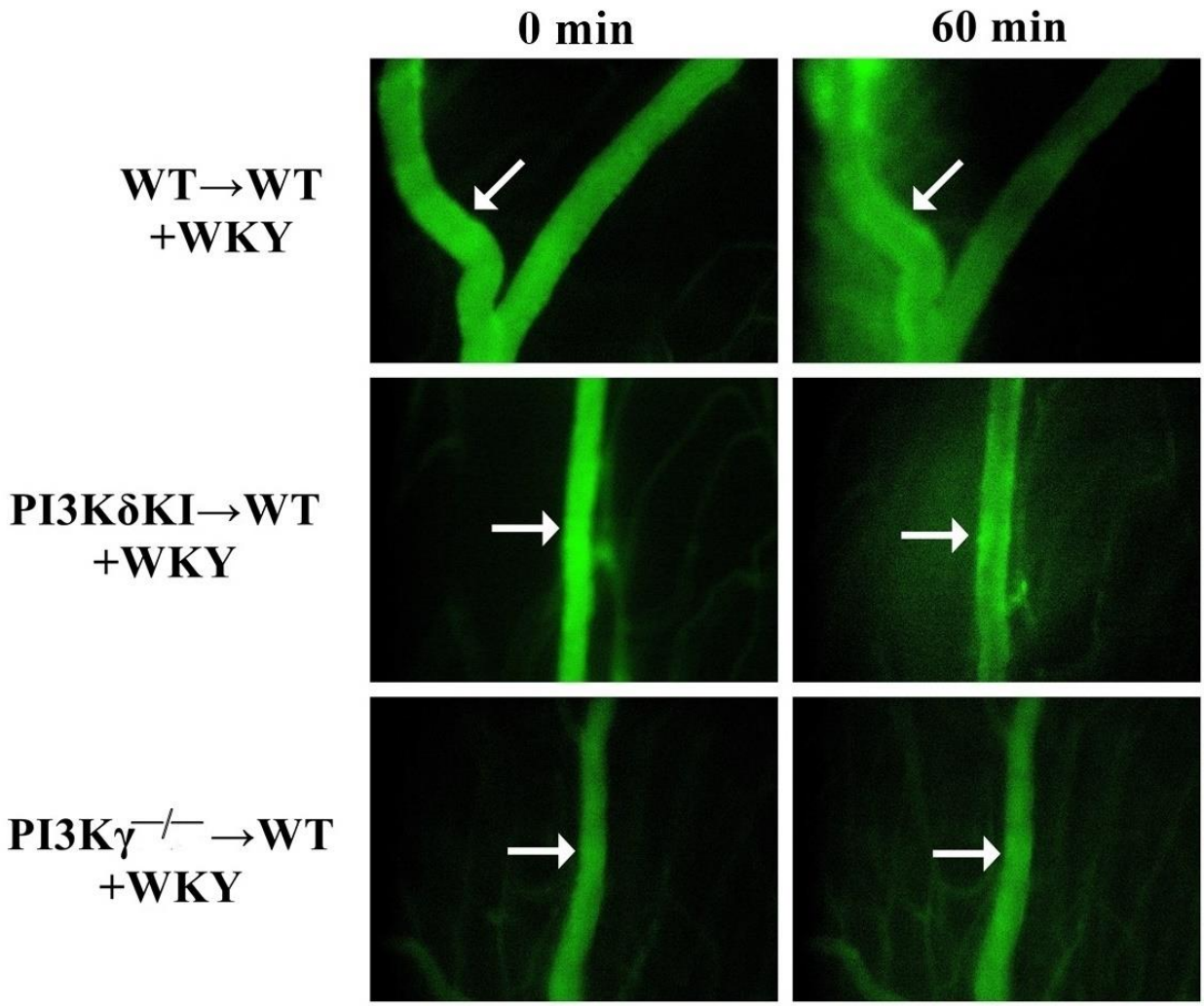

Figure 3-7. WKYMVm-induced microvascular hyperpermeability and neutrophil recruitment in PI3Kγ or PI3Kδ chimeric mice (micrographs). Representative fluorescence intravital micrographs of mouse cremasteric post-capillary venules were taken before (0 min) and 60 min after injection of FITC-labelled BSA (25 mg/kg, i.v.) in WT→WT mice (upper panel), PI3K$\delta^{D910A/D910A}$ (PI3KδKI)→WT mice (middle panel) and PI3K$\gamma^{-/-}$→WT mice (lower panel). Arrow in micrograph indicates the venular segment observed for permeability index measurements (Magnification: ×20).

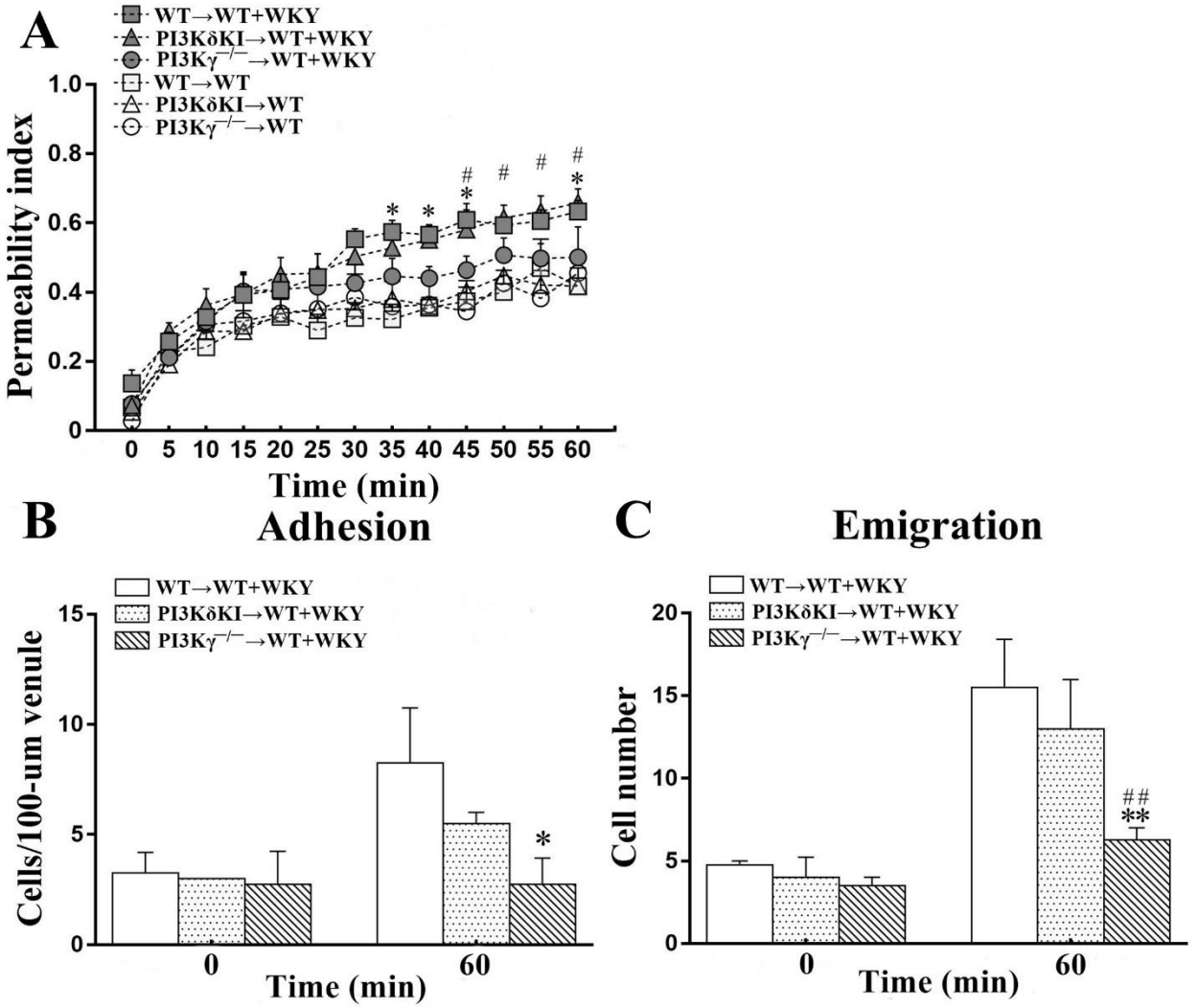

*Figure 3-8. WKYMVm-induced microvascular hyperpermeability and neutrophil recruitment in PI3Kγ or PI3Kδ chimeric mice (quantification results). (A) Permeability index of mouse cremasteric post-capillary venules was measured following 1-h superfusion of bicarbonate-buffered saline (white symbols, n=3) or WKYMVm (WKY, 0.1 μM, grey symbols, n=4) in PI3Kγ or PI3Kδ chimeric mice. Mean ± SEM. Adhesion cell number of 100-μm length of venule (B) and emigration cell number (cells/443×286 μm² field, C) were determined simultaneously in the same venule for WKYMVm-induced cremasteric microvascular hyperpermeability in PI3Kγ or PI3Kδ chimeric mice before (0 min) and 60 min following WKYMVm superfusion. A–C, mean ± SEM of 4 mice per group. * and ** indicate significant differences ($p<0.05$ and $p<0.01$, respectively) between WT→WT mice and PI3Kγ$^{-/-}$→WT mice. # and ## indicate significant differences ($p<0.05$ and $p<0.01$, respectively) between PI3Kδ$^{D910A/D910A}$→WT mice and PI3Kγ$^{-/-}$→WT mice.*

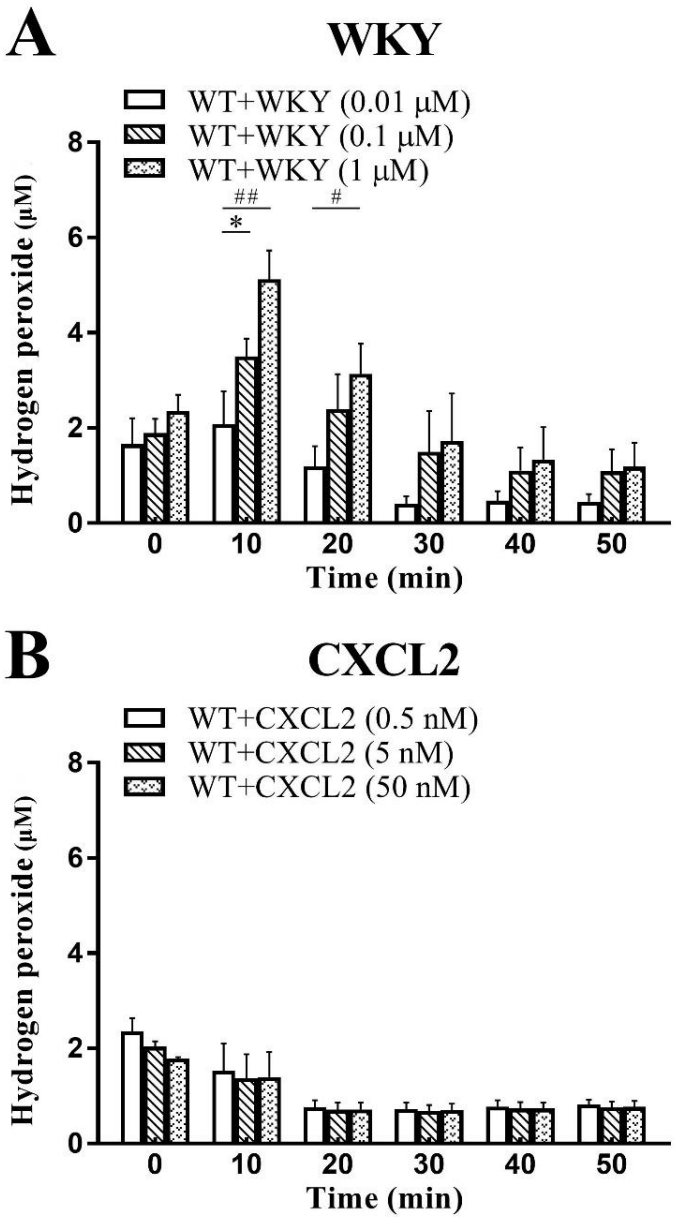

*Figure 3-9. Dose-dependent responses of WKYMVm and CXCL2 on extracellular hydrogen peroxide generation from wild-type neutrophils. Neutrophils were freshly isolated from the bone marrows of wild-type (WT) mice and stimulated with varied doses of WKYMVm (WKY, 0.01–1 μM, A) or CXCL2 (0.5–50 nM, B) for extracellular hydrogen peroxide determination as described in Material and methods. A–B, mean ± SEM of 3 mice per group. The measurements were duplicated in the 96-well plate. * indicates significant differences ($p<0.05$) between WKYMVm doses of 0.01 μM and 0.1 μM. # and ## indicate significant differences ($p<0.05$ and $p<0.01$, respectively) between WKYMVm doses of 0.01 μM and 1 μM.*

3.5.7 The catalytic function of PI3Kγ is required in WKYMVm-induced generation of extracellular ROS in neutrophils

A series of experiments were performed to determine the extracellular production of hydrogen peroxide in bone marrow-derived neutrophils from WT, PI3Kγ$^{-/-}$ and PI3Kδ$^{D910A/D910A}$ mouse strains. Isolated neutrophils were stimulated with 0.1 μM WKYMVm and the time-dependent production of extracellular hydrogen peroxide was determined. Starting from 20 min, deficiency of PI3Kγ strongly impaired WKYMVm-stimulated production of extracellular hydrogen peroxide in neutrophils (Figure 3-10A). This WKYMVm-stimulated response was not significantly changed in neutrophils from PI3Kδ-mutant mice as compared with those from the WT control mice, but the response in PI3Kδ-mutant neutrophils was significantly higher than PI3Kγ$^{-/-}$ neutrophils at 40 min and 50 min. These results provide clear evidence that WKYMVm-stimulated production of extracellular hydrogen peroxide is impaired in PI3Kγ$^{-/-}$ but not PI3Kδ-mutant neutrophils.

To address whether the generation of ROS in neutrophils is through structural functions or catalytic functions of PI3Kγ, we pre-treated the isolated neutrophils with the highly specific PI3Kγ inhibitor CZC24832. As shown in Figure 3-10B, the pre-incubation of CZC24832 eliminated the differences among PI3Kγ$^{-/-}$ neutrophils and the other two types of neutrophils. These results suggest that WKYMVm-induced PI3Kγ-dependent ROS production is due to PI3Kγ-kinase-dependent function. Moreover, two ROS inhibitors, DPI and MnTBAP, also eliminated the differences in WKYMVm-induced ROS generation among three types of neutrophils (Figure 3-10C and 3-10D).

3.5.8 ROS scavenging attenuates PI3K isoform-specific differences in WKYMVm-induced microvascular hyperpermeability

We applied the ROS inhibitors to WKYMVm-superfused mouse cremaster muscle to identify the role of ROS in PI3Kγ-dependent leakage in the local microvascular barrier. As depicted in Figure 3-11A, pre-treatment with DPI resulted in reduced permeability index curve for

PI3K$\delta^{D910A/D910A}$ mice and eliminated the differences in microvascular hyperpermeability between PI3K$\gamma^{-/-}$ mice and PI3K$\delta^{D910A/D910A}$ mice but maintained the differences in the change of permeability between PI3K$\gamma^{-/-}$ mice and WT mice (albeit from 35 min and onwards of WKYMVm superfusion). By contrast, pre-superfusion with MnTBAP not only eliminated the differences of WKYMVm-stimulated permeability among all three mouse strains but also suppressed this hyperpermeability response altogether (Figure 3-12A). We further observed the effects of DPI and MnTBAP on the number of adherent and emigrated neutrophils after 60 min superfusion of WKYMVm. With DPI treatment (Figure 3-11B and 3-11C), the adhesion and emigration responses in WT mice were partially impaired, and PI3Kγ or PI3Kδ deficiency still had significantly reduced neutrophil adhesion but the emigration was similar to the WT mice under this condition. With MnTBAP treatment (Figure 3-12B and 3-12C), the deficiency of PI3Kγ still showed a significant reduction in both neutrophil adhesion and emigration compared to those in WT mice, whereas loss-of-function of PI3Kδ only reduced neutrophil emigration under this condition of reduced ROS. These data indicate that either the NADPH oxidase inhibitor or the SOD mimic is capable of eliminating WKYMVm-induced PI3Kγ-dependent microvascular hyperpermeability increases as well as PI3Kγ-dependent neutrophil emigration.

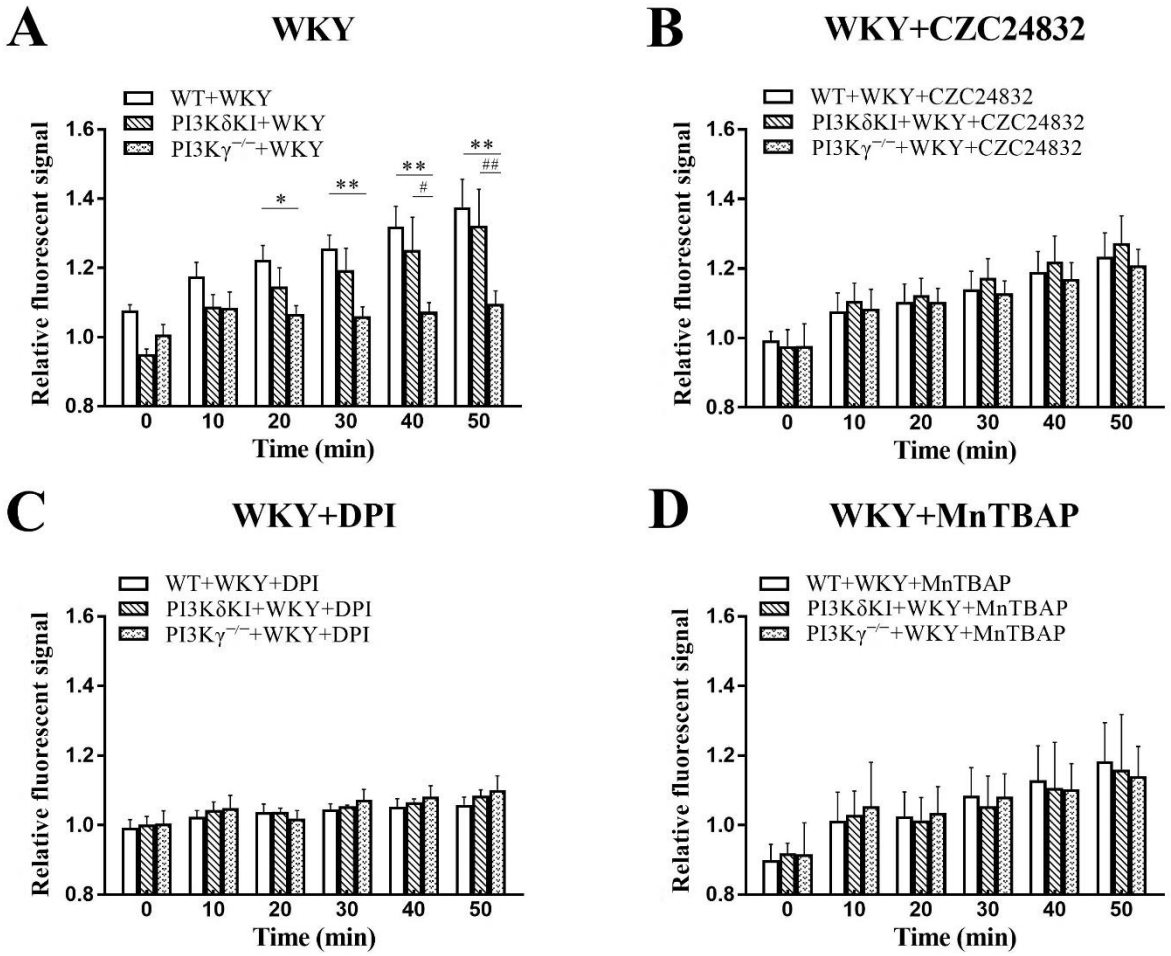

*Figure 3-10. Time course of WKYMVm-induced extracellular hydrogen peroxide generation from wild-type, PI3K$\delta^{D910A/D910A}$ and PI3K$\gamma^{-/-}$ neutrophils. Neutrophils were freshly isolated from bone marrows of wild-type (WT), PI3K$\delta^{D910A/D910A}$ (PI3KδKI) and PI3K$\gamma^{-/-}$ mice and stimulated with WKYMVm (WKY, 0.1 μM) and with pre-treatment of blank Krebs-Ringer phosphate glucose buffer (A), CZC24832 (PI3Kγ inhibitor, 10 μM, B), DPI (NADPH oxidase inhibitor, 40 μM, C) or MnTBAP (SOD mimic, 200 μM, D) for extracellular hydrogen peroxide determination as described in Material and methods. A–D, mean ± SEM of 3 mice per group. The measurements were duplicated in the 96-well plate. * and ** indicate significant differences ($p<0.05$ and $p<0.01$, respectively) between WT neutrophils and PI3K$\gamma^{-/-}$ neutrophils. # and ## indicate significant differences ($p<0.05$ and $p<0.01$, respectively) between PI3K$\delta^{D910A/D910A}$ neutrophils and PI3K$\gamma^{-/-}$ neutrophils.*

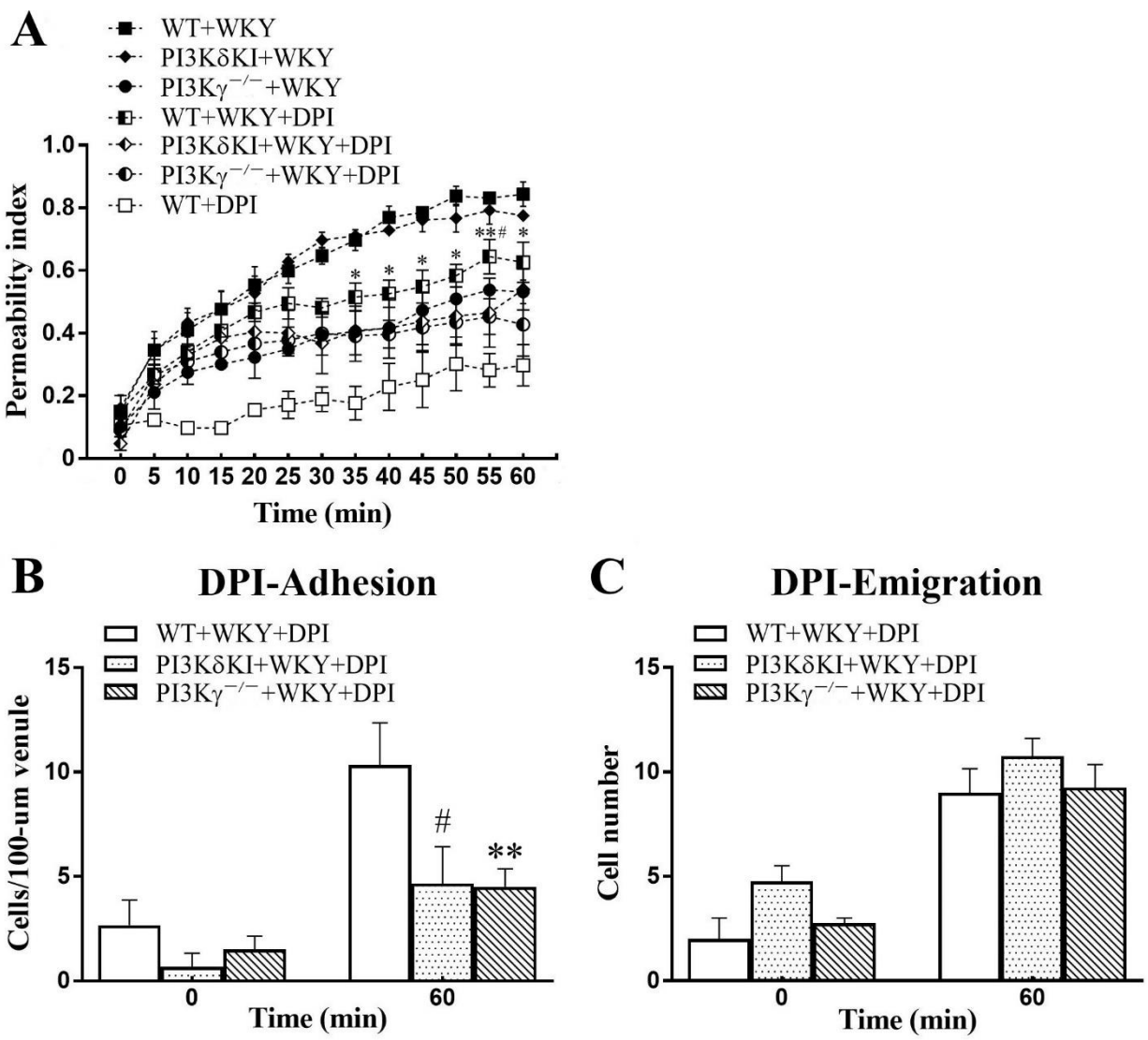

*Figure 3-11. Effect of DPI on WKYMVm-induced microvascular hyperpermeability and neutrophil recruitment in wild-type, PI3K$\delta^{D910A/D910A}$ and PI3K$\gamma^{-/-}$ mice. Permeability index of mouse cremasteric post-capillary venules was measured following 1-h WKYMVm (WKY, 0.1 μM) superfusion in the absence or presence of DPI (NADPH oxidase inhibitor, 40 μM, A) for 30 min prior to and during 1-h WKYMVm superfusion. Adhesion cell num ber of 100-μm length of venule (B) and emigration cell number (cells/443×286 μm^2 field, C) were determined simultaneously in the same venule of WKYMVm-induced cremasteric microvascular hyperpermeability in the presence of DPI (B and C). A–C, mean ± SEM of 4 mice per group. * and ** indicate significant differences ($p<0.05$ and $p<0.01$, respectively) between wild-type (WT) mice and PI3K$\gamma^{-/-}$ mice. # indicates significant differences ($p<0.05$) between WT mice and PI3K$\delta^{D910A/D910A}$ (PI3KδKI) mice.*

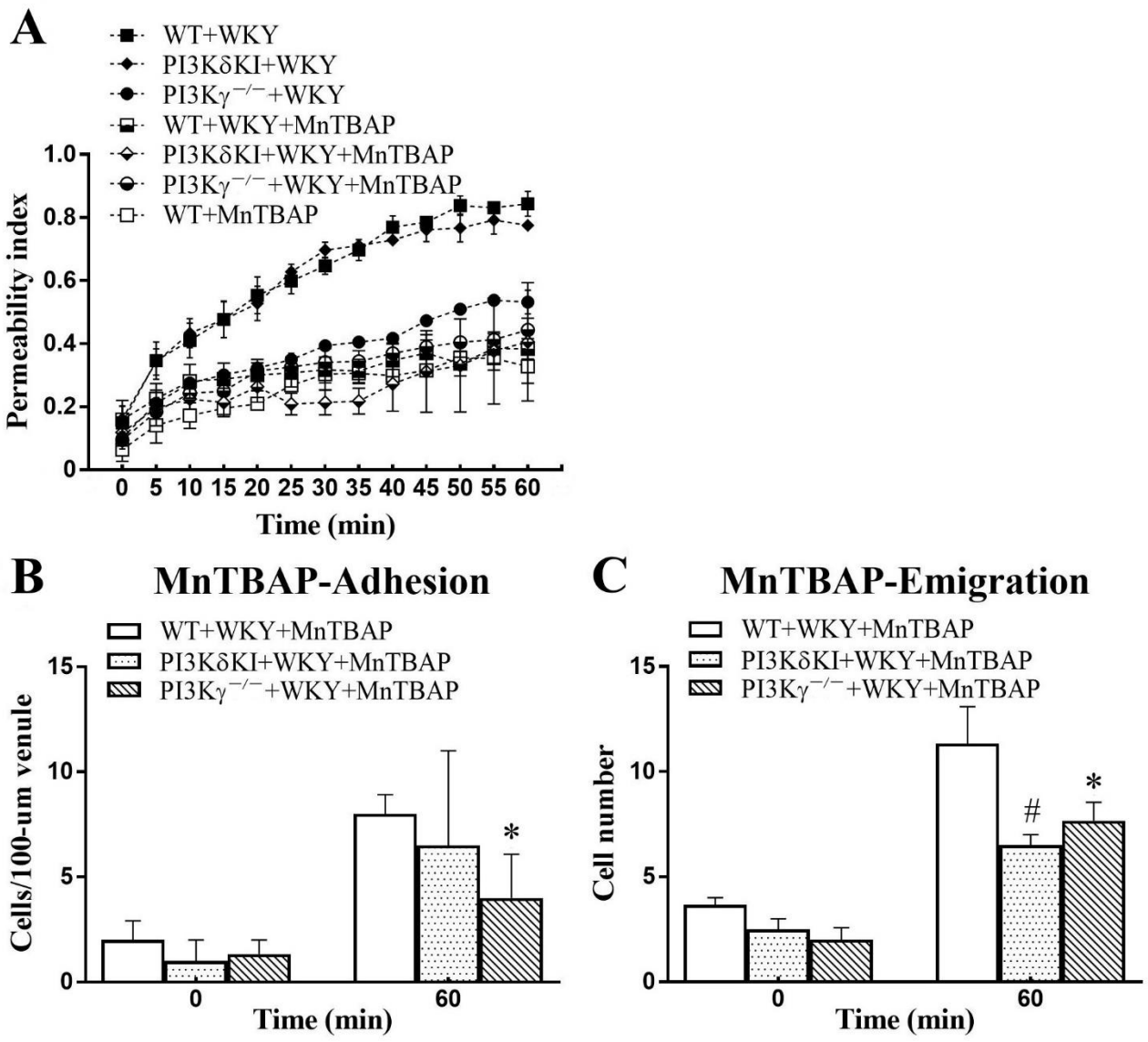

*Figure 3-12. Effect of MnTBAP on WKYMVm-induced microvascular hyperpermeability and neutrophil recruitment in wild-type, PI3K$\delta^{D910A/D910A}$ and PI3K$\gamma^{-/-}$ mice. Permeability index of mouse cremasteric post-capillary venules was measured following 1-h WKYMVm (WKY, 0.1 μM) superfusion in the absence or presence of MnTBAP (SOD mimic, 200 μM, A) for 30 min prior to and during 1-h WKYMVm superfusion. Adhesion cell number of 100-μm length of venule (B) and emigration cell number (cells/443×286 μm^2 field, C) were determined simultaneously in the same venule of WKYMVm-induced cremasteric microvascular hyperpermeability in the presence of MnTBAP (B and C). A–C, mean ± SEM of 4 mice per group. * indicates significant differences ($p<0.05$) between wild-type (WT) mice and PI3K$\gamma^{-/-}$ mice. # indicates significant differences ($p<0.05$) between WT mice and PI3K$\delta^{D910A/D910A}$ (PI3KδKI) mice.*

3.6 Discussion

In this study, we demonstrate a PI3Kγ-dependent and neutrophil ROS generation mechanism in formyl peptide receptor agonist WKYMVm-induced but not chemokine CXCL2-induced microvascular hyperpermeability during neutrophil recruitment. Since mounting evidence has been demonstrated that the endothelium *per se* is involved in local oxidative stress in microvascular barrier impairment [325,326], we carefully excluded the contribution of endothelial PI3Kδ and PI3Kγ isoforms by using PI3K chimeric mice in this model of microvascular hyperpermeability. Our results strongly indicate that PI3Kγ-dependent microvascular hyperpermeability during WKYMVm-induced neutrophil recruitment is largely due to PI3Kγ-dependent production of reactive oxygen species by the recruiting neutrophils.

It was reported that formyl tripeptide fMLP substantially increased microvascular permeability [327], but the effects of WKYMVm, a mimicry of fMLP, on mouse microvascular leakage remains unclear. Our current study demonstrates a proinflammatory role of WKYMVm in the barrier functions of mouse post-capillary venules. Moreover, chemokine CXCL2 also increased, to a lesser extent than WKYMVm, the microvascular hyperpermeability in mouse cremaster muscle, which is in line with the previous studies in mouse lung disease [328,329]. Interestingly, although both the end-target chemoattractant WKYMVm and the intermediary chemoattractant CXCL2 induced a rapid elevation in mouse microvascular permeability, the underlying mechanisms may be very different. Our observation of a differential requirement for PI3Kγ in hyperpermeability in WKYMVm-treated but not CXCL2-treated murine cremasteric venules strongly suggests that a PI3Kγ-dependent mechanism is contributing to the WKYMVm-induced but not CXCL2-induced microvascular hyperpermeability.

Beyond many other factors, the neutrophil is an active cell type in regulating vascular permeability [330]. Neutrophils increase microvascular leakage during recruitment through their physical interactions with the endothelium at the circulation and tissue interfaces during neutrophil adhesion and transmigration [331,332]. They also act through local production of inflammatory mediators including oxidants [333], cytokines [334], lipid metabolites [335], leukotrienes [100]

and proteases [336]. In this study, the PI3Kγ-dependent mechanism in microvascular hyperpermeability is dissipated after neutrophil depletion in mouse circulation. This clearly demonstrates that WKYMVm-induced, PI3Kγ-dependent microvascular hyperpermeability is neutrophil-driven. This conclusion is further supported by our experimental results of transplanting bone marrow for neutrophil generation from different PI3K transgenic mouse strains into the mice with the same WT endothelial background. Although we cannot exclude the effects of other mediators in the total elevation of microvascular permeability, our work proves that a signaling function of PI3Kγ within neutrophils plays a predominant role in WKYMVm-induced microvascular permeability elevation in inflammation.

The involvement of neutrophil adhesion and emigration in promoting microvascular leakage is an inevitable and coincidental event. It has been generally accepted that neutrophil adhesion and emigration cause endothelium leakage through many different mechanisms, such as increasing intracellular Ca^{2+}, producing ROS and inducing actin cytoskeleton changes in endothelial cells [117]. However, our data reveal that the role of PI3Kγ in mediating microvascular leakage may be uncoupled from the changes in neutrophil adhesion and emigration. In our study, two lines of experimental evidence support this notion: 1) similar degrees of reduction in adhesion and emigration were observed in the two strains of PI3K-deficient mice in both WKYMVm- and CXCL2-treated experimental models, but only in WKYMVm-treated model was there PI3Kγ-dependent discrepancy in hyperpermeability; 2) after scavenging the local ROS production in WKYMVm-treated model, the persisting significant gaps in the number of adhesive and emigrated cells among mouse strains were insufficient to trigger PI3Kγ-dependent discrepancy in vascular hyperpermeability. This uncoupling of adhesion and emigration to vascular hyperpermeability was also reported in some previous studies. In leukotriene B_4-stimulated human lungs, transmigrating neutrophils infiltrated into the alveolar spaces without increasing microvascular permeability [337]. Vascular leakage during neutrophil transendothelial migration can be minimized by the formation of endothelial dome structure (also known as the transmigratory cup), an endothelial subcellular structure that encapsulates the transmigrating neutrophils [32,38]. Therefore, as shown in this study

and elsewhere [227], PI3Kγ has a critical role in neutrophil adhesion and emigration which are important for the dynamic neutrophil-endothelium interacting processes and for the elevation of microvascular permeability. Our study demonstrates that the PI3Kγ-dependent mechanism in WKYMVm-induced microvascular leakage involves other PI3Kγ-related factors rather than solely neutrophil adhesion and emigration.

PI3Kγ is required for the production of ROS in fMLP-treated human neutrophils [338]. The G-protein β and γ subunits and the Ras binding domain of p110γ, the catalytic subunit of PI3Kγ, conduct PI3K signaling by binding to the regulatory subunits p84 and p101 of PI3Kγ [339]. The distinct roles of p84 and p101 were demonstrated in recent years: p101 functions in PI3Kγ-mediated migration, whereas p84 functions in PI3Kγ-dependent ROS generation [340]. When neutrophils are stimulated with fMLP, the G-protein-coupled receptors on the cell membrane deliver the activating signal through PI3Kγ isoform. Also, PKCα is activated by forming the PI3Kγ-PKCα complex *via* an unknown mechanism that p84 is involved in. The activation of both PI3Kγ and PKCα signaling pathways increases their downstream enzymatic activities, which result in the generation of ROS in neutrophils [341]. In this regard, our observation of PI3Kγ-dependent ROS generation in WKYMVm-treated mouse neutrophils is consistent with the conclusions in previous studies. In addition, in line with previous studies which indicated a possible role of PI3Kδ in maximizing ROS production in the second phase of respiratory burst [338], the role of PI3Kδ in extracellular ROS generation in mouse neutrophils was also indicated in our study, albeit not as crucial as the role of PI3Kγ.

The local oxidative stress in the microvasculature is an essential factor that increases microvascular leakage [342]. In our study, locally scavenging ROS by MnTBAP completely abolished the difference between WT and PI3Kγ-deficient mice in WKYMVm-induced microvascular permeability, whereas scavenging DPI only reduced the difference between the two PI3K transgenic mouse strains. It has been known that DPI is a potent and reversible inhibitor that simultaneously inhibits ROS generation from neutrophil NADPH oxidase on the membrane as well as superoxide and H_2O_2 production through NADH-ubiquinone oxidoreductase (complex I) in

mitochondria [343]. However, MnTBAP is a cell-permeable superoxide dismutase mimetic and acts as a ROS scavenger to increase the metabolism of superoxide. We speculate that the different effects of two inhibitors may be due to 1) the small portion of ROS derived from minor sources (peroxisomes and endoplasmic reticulum) since DPI only inhibits two major sources whereas MnTBAP metabolizes ROS regardless of their sources, and 2) the role of peroxynitrite because commercial MnTBAP (purity usually >96%, but <100%) may scavenge peroxynitrite as well as ROS [344]. Further studies are needed to determine where and which free radical species of ROS are critical in inducing microvascular hyperpermeability during neutrophil recruitment.

Taken together, our results suggest that PI3Kγ-dependent generation of ROS in neutrophils is critically important in WKYMVm-induced hyperpermeability in post-capillary venules during neutrophil recruitment. This reveals a mechanism involving PI3Kγ-specific signaling pathway in regulating the increases of microvascular permeability in acute inflammation where formyl peptides are engaged.

CHAPTER FOUR

SUPPRESSIVE ROLE OF BAM32 IN CHEMOKINE-INDUCED NEUTROPHIL RECRUITMENT THROUGH RAP1 ACTIVATION

4.1 Preface

In this chapter, we uncover a novel role for Bam32 in CXCL2-induced neutrophil transmigration and chemotaxis through modulating the activation of downstream small GTPase Rap1 which further suppresses the phosphorylation of Akt. This study, for the first time, shows a mechanism of the Bam32/Rap1/Akt signaling pathway in regulating neutrophil recruitment in acute inflammation.

Data presented in this chapter has been included in a research paper by Li Hao, Aaron J. Marshall and Lixin Liu in a peer-review scientific journal. The contents of this chapter have been adapted from the manuscript. In this study, Li Hao conducted all the experiments, performed data analysis, drafted the manuscript, and participated in the study design, data interpretation and manuscript revision.

4.2 Abstract

Bam32 (B cell adaptor molecule of 32 kDa) functions in immune responses of various leukocytes. However, the role of neutrophil Bam32 in inflammation is entirely unknown. Here, we determined the role of Bam32 in chemokine CXCL2-induced neutrophil chemotaxis in three mouse models of neutrophil recruitment (the cremaster muscle model, the peritonitis model, and the 2D-chemotaxis model). By using intravital microscopy in mouse cremaster muscle, we found that transmigrated neutrophil number, neutrophil chemotaxis velocity and total neutrophil chemotaxis distance were increased in Bam32$^{-/-}$ mice when compared with wild-type (WT) mice. In CXCL2-induced mouse peritonitis, the total emigrated neutrophils were increased in Bam32$^{-/-}$ mice at 2 h but not 4 h. CXCL2-induced chemotaxis distance and migration velocity of isolated Bam32$^{-/-}$ neutrophils *in vitro* were increased. We examined the activation of small GTPases Rac1 and Rap1, the expression levels of phospho-Akt2 and total Akt2 and their crosstalk with Bam32 in neutrophils. The deficiency of Bam32 resulted in the elevation of Rac1-GTP binding and suppression of Rap1-GTP binding. Pharmacological inhibition of Rac1 by NSC23766 markedly reduced activation of Rap1 in WT neutrophils and reduced Bam32$^{-/-}$ neutrophil chemotaxis, whereas inhibition of Rap1 by GGTI298 increased WT neutrophil chemotaxis. In addition, deficiency of Bam32, as well as inhibition of Rap1 activation, increased levels of CXCL2-induced Akt1/2 phosphorylation at Thr308/309 in neutrophils. Inhibition of Akt by SH-5 attenuated CXCL2-induced adhesion and emigration in Bam32$^{-/-}$ mice. Together, our results reveal a suppressive role for Bam32 in regulating chemokine-induced neutrophil migration and chemotaxis *in vivo* and *in vitro*. This Bam32-dependent suppression is mediated by Rap1 activation through suppressing Akt phosphorylation in neutrophils.

4.3 Introduction

Neutrophil recruitment is a dynamic multi-step process that is regulated by a variety of molecules and signaling cascades elicited by activation of Gi-coupled chemoattractant and chemokine receptors during acute inflammation [21]. A substantial body of evidence *in vivo* and *in vitro* indicates that PI3K, such as Class IB isoform PI3Kγ, plays a critical role in the initial signal transduction downstream of the chemokine receptors [43,219,222]. Upon stimulation by the majority of chemoattractants, PI3Kγ increases localized accumulation of membrane-captive phospholipid PI(3,4,5)P3 which directs neutrophil movement and remodeling through binding to the recruited PI3K adaptor proteins at the leading edge on the cell membrane [219]. The affinity of this interaction may vary: some signaling proteins such as Grp1 and Btk preferentially bind PI(3,4,5)P3 [345,346], some signaling proteins such as TAPP1/2 preferentially bind PI(3,4)P2 [253], and some others such as Akt bind both [347]. Although the functions of a few phosphoinositide-binding proteins such as Akt were well studied previously, the roles for most of the rest adaptor proteins remain unclear.

B cell adaptor molecule of 32 kDa (Bam32), also known as dual adaptor for phosphotyrosine and 3-phosphoinositides 1 (DAPP1), is one of the PI3K adapter proteins that interact with PI(3,4,5)P3 and the breakdown product PI(3,4)P2, regulating downstream cell growth, proliferation, and differentiation [263]. Bam32 is widely expressed in leukocytes including B lymphocytes, T lymphocytes, mast cells and dendritic cells (DC) [257], in which the roles of Bam32 were uncovered in recent years. In mouse B lymphocytes, Bam32 induces the cytoskeletal rearrangement, actin remodeling, membrane ruffling, and lamellipodium formation by regulating Rac1 activation [266]. It also increases B cell adhesion and spreading on integrin ligands through the activation of Rho family GTPases [269]. Bam32 in mast cells inhibits FcεRI-induced calcium flux and granule release by activating Lyn and SHIP negative signaling pathways [261]. Furthermore, Bam32 in DC is located at the DC–T cell contact sites and binds to galectin-1 to suppress $FOXP3^{+}$-induced proliferation of T cells [262]. Bam32 is expressed at similar levels in

mouse neutrophils as in B cells [348]; however, the role of Bam32 in neutrophils remains completely undefined.

In this study, we investigated the role of Bam32 in mouse neutrophil migration and chemotaxis both *in vivo* and *in vitro*. By using real-time intravital microscopy and time-lapsed movie analysis, we examined chemokine CXCL2-induced neutrophil transmigration and chemotaxis in cremaster muscle and neutrophil recruitment in the peritoneum of $Bam32^{-/-}$ mice. Moreover, we determined CXCL2-induced chemotaxis in isolated $Bam32^{-/-}$ neutrophils. By analyzing the activation of small GTPases Rac1 and Rap1, phosphorylation of Akt in CXCL2-stimulated neutrophils, and the effects of corresponding inhibitors in both *in vivo* and *in vitro* models, we unraveled the underlying Bam32-dependent mechanisms involved in CXCL2-induced transmigration and chemotaxis of neutrophils in mice.

4.4 Material and methods

4.4.1 Animals

Bam32$^{-/-}$ mice were generated by Dr. Michel C. Nussenzweig [263], transferred to and maintained in the Lab Animal Services Unit at the University of Saskatchewan (Saskatoon, SK, Canada). Age-matched male wild-type (WT) C57BL/6N mice were purchased from Charles River Canada (Saint-Constant, QC, Canada). Male mice of these two strains were used between 6 and 12-week-old. This study was carried out with the approval of animal protocols from the University Committee on Animal Care and Supply at the University of Saskatchewan and following the standards of the Canadian Association of Animal Care. All efforts were made to reduce animal suffering and all surgeries were performed under deep ketamine-xylazine anesthesia.

4.4.2 Polymerase chain reaction

Bam32$^{-/-}$ mice carry a Bam32 gene in which exon 1 is replaced by a neomycin resistance cassette [263]. Ear tissue was collected from each breeding parent of Bam32$^{-/-}$ mice. DNA samples were extracted with Wizard Genomic DNA Purification Kit (Promega, Madison, WI), followed by being amplificated with GoTaq PCR Core System (Promega) and the following primers:

5'-CCTCTTACTCACAGCCGTGTAGT-3' (Common forward)

5'-CGAGCACGTACTCGGATGGAAG-3' (Bam32KO reverse)

5'-ATGGGCAGAGCAGAACTTCTAGGA-3' (WT reverse)

All primers were provided by Integrated DNA Technologies (Coralville, IA). A PCR product of approximately 500 bp represents the WT allele, while a product of approximately 800 bp represents the knock-out allele, as described previously [263].

4.4.3 Intravital microscopy

Mice were anesthetized after intraperitoneal injection with a cocktail of ketamine (200 mg/kg, Roger, Montreal, QC, Canada) and xylazine (10 mg/kg, Bayer, Toronto, ON, Canada). The mouse cremaster muscle was surgically exposed as described previously [312]. The exposed cremaster

muscle was superfused with 37°C-warmed bicarbonate-buffered saline (pH 7.4; containing in mM, NaCl 133.9, KCl 4.7, $MgSO_4$ 1.2, and $NaHCO_3$ 20.0; all reagents purchased from Thermo Fisher Scientific, Toronto, ON, Canada). An upright microscope (model Eclipse Ci-s, Nikon, Tokyo, Japan) with a 20× objective lens was used for bright-field intravital microscopy. A 2% agarose gel of 1-mm^3 size containing murine CXC chemokine CXCL2 (0.5 μM, R&D Systems, Minneapolis, MN) was placed on the exposed cremaster muscle in a preselected area 350-μm distant from and parallel to the observed postcapillary venule. After placing a 22 mm × 22 mm glass coverslip to hold the gel, the cremaster muscle was superfused with 37°C-warmed bicarbonate-buffered saline at a very slow rate (≤ 10 μl/min) to allow the formation of CXCL2 chemotactic gradient. Throughout the experiment, neutrophil recruitment and hemodynamic changes in the selected cremasteric postcapillary venule (25–35 μm diameter) were recorded at real-time on a DVD video recorder upon (0 min) and after addition of CXCL2-containing gel (recorded for 60 min). During recording, all efforts were made to adjust and keep the microscopic images focused on the adhering, crawling, transmigrating and chemotactic neutrophil inside the venule and in extravascular cremaster muscle tissue. The numbers of adherent and transmigrated neutrophils were determined in cremasteric microvasculature during offline playback analysis of the recorded real-time video. The emigrated neutrophils were defined as the neutrophils emigrated to the extravascular tissue from the venule, excluding the neutrophils that attached on the tunica media. The number of emigrated neutrophils in intravital microscopy was counted in the field of 221×286 μm^2 (one full screen on the monitor) adjacent to the recorded venule. A 512× time-lapsed movie (sped up by 512-fold) was made from the 1-h real-time recording. The crawling distance, crawling velocity, transendothelial time, detachment time, migration distance, migration velocity, chemotaxis distance, chemotaxis velocity, and chemotaxis index were analyzed in the time-lapsed movie as described previously [314,315]. Briefly, the crawling distance is the total distance (μm) traversed by an average crawling neutrophil on the luminal surface of the endothelium. The crawling velocity is the velocity (μm/min) of neutrophil crawling on the luminal surface of the endothelium. The transendothelial time is the duration (min) of neutrophil transmigration across the endothelium. The detachment time is the

detachment time (min) of transmigrated neutrophils from the extravascular surface of the venule. The migration distance is the total distance (μm) migrated by an average chemotactic neutrophil in extravascular tissue. The migration velocity is the velocity (μm/min) of neutrophil migration in extravascular tissue. The chemotaxis distance is the total chemotactic distance (μm) in the direction to the source of CXCL2 but perpendicular to the venule migrated by a chemotactic neutrophil in extravascular tissue. The chemotaxis velocity is the velocity (μm/min) in the direction of the source of CXCL2. The chemotaxis index is the ratio of dividing the chemotaxis distance by the migration distance. Where indicated, the specific Akt inhibitor SH-5 (1 μM, Santa Cruz Biotechnology, Inc., Mississauga, ON, Canada) was superfused on the cremaster muscle 30 min prior to and throughout 1-h CXCL2 stimulation.

4.4.4 Cell counting in CXCL2-induced acute peritonitis

Emigrated neutrophils were harvested with ice-cold lavage solution (pH 7.35; bovine serum albumin 2.5% w/v, in PBS) from the mouse peritoneum at 2 h or 4 h after intraperitoneal injection of CXCL2 (0.5 μg/25 g body weight) or glycogen from oyster (10mg/25 g in sterile saline, Sigma-Aldrich, Oakville, ON, Canada), and were centrifuged at 300× *g* and 4°C for 5 min. The cell pellet was resuspended with 1 ml ice-cold cytospin solution (containing bovine serum albumin 10% w/v and NaN_3 3.1 mM, in saline). Cell suspension (1×10^6 mL, 150 μL) was loaded into the cytospin chamber. A film of cells on slides was prepared by cytocentrifugation, fixed and stained with Richard-Allan staining solutions (Thermo Fisher Scientific). The numbers of total emigrated leukocytes and emigrated neutrophils were morphologically quantified under a bright-field microscope (Magnification: ×200).

4.4.5 Harvest of oyster glycogen-induced emigrated neutrophils

Neutrophils were isolated from oyster glycogen-induced acute peritonitis model of WT and $Bam32^{-/-}$ mice respectively. Glycogen from oyster (10mg/25 g in sterile saline, Sigma-Aldrich) was injected into mouse peritoneum 4 h before emigrated neutrophils were harvested with ice-cold

lavage solution from the mouse peritoneum.

4.4.6 Harvest of neutrophils from mouse bone marrow

Neutrophils were freshly isolated from the bone marrow of WT and Bam32$^{-/-}$ mice respectively. The femur and tibia were dissected immediately after the mouse was euthanized by cervical dislocation. The marrow cavity was flushed with ice-cold calcium- and magnesium-free PBS. The bone marrow cells enriched in flushing fluid were separated by three-density (72%, 64%, and 52%) Percoll (GE Healthcare, Baie d'Urfe, QC, Canada) gradient centrifugation at 1080× *g* for 30 min as described previously [319]. This method yielded over 85% morphologically mature neutrophils [320].

4.4.7 Two-dimensional (2D) chemotaxis assay in vitro

Time-lapse video microscopy was used to determine the distance, speed, and directionality of neutrophil chemotaxis in response to the CXCL2 chemokine gradient using a previously described method [349]. Primary neutrophils from either fresh bone marrow or emigrated neutrophils from oyster glycogen-induced peritonitis were tested on uncoated ibiTreat Chemotaxis 2D µ-slides (ibidi, Munich, Germany). Isolated neutrophils suspended in 37°C Dulbecco's Modified Eagle Medium (DMEM; ATCC, Manassas, VA) containing 10% FBS were seeded into the narrow channel of the µ-slide strictly following the manufacturer's user manual. After 30 min incubation in a humidified incubator at 37°C with 5% CO_2, CXCL2 (18 µL, 6.25 µM) was loaded into one of the two opposing reservoirs, which were filled with cell-free medium, to allow the formation of the concentration gradient at the observation area. The chemotactic movements of the adherent neutrophils were recorded for 60 min at 37°C using an ibidi heating and incubation chamber on an inverted microscope (Applied Precision) equipped with a CCD color video camera. Time-lapsed movie analysis of chemotaxis was performed using ImageJ software (National Institutes of Health, Bethesda, MD), and at least 50 cells were tracked and analyzed for each group. Where indicated, Rac1 inhibitor NSC23766 (50 µM, Tocris, Oakville, ON, Canada) or Rap1

inhibitor GGTI298 (10 μM, Tocris, Oakville, ON, Canada) was incubated with neutrophils for 30 min prior to loading onto the μ-slide and remained throughout 1-h CXCL2 stimulation.

4.4.8 Rac1 activation assay

Fresh bone marrow-derived mouse neutrophils were prepared as described above and suspended in 37°C serum-free DMEM (ATCC). Colorimetric Rac1 G-LISA Activation Assay Kit (Cytoskeleton, Inc., Denver, CO) was used to quantitatively detect GTP-bound Rac1 after 2-min stimulation of neutrophils in suspension with CXCL2 (0.1 μM) at 37°C. All steps of lysing neutrophils and extracting GTP-bound Rac1 were performed strictly following the instruction provided by the manufacturer. Protein concentrations were determined using the Precision Red Advanced Protein Assay that came with the kit. The fluorescent signal of each sample on 96-well plates was captured by Fluoroskan Ascent Microplate Fluorometer (Thermo Fisher).

4.4.9 Rap1 pull-down assay

Fresh bone marrow-derived mouse neutrophils were prepared as described above and suspended in 37°C serum-free DMEM. After 2-min stimulation of neutrophils with CXCL2 (0.1 μM) at 37°C, total Rap1 and GTP-bound Rap1 in neutrophils were determined using Active Rap1 Detection Kit (Cell Signalling Technology, New England Biolabs, Ltd., Whitby, ON, Canada) according to the manufacturer's instruction. Briefly, total protein lysates were incubated with GST-bound RalGDS-RBD at 4°C for 1 h. The resin was then washed and incubated with 2× Laemmli sample buffer to elute GTP-bound Rap1. The elutes and total protein lysates were electrophoresed in 12% SDS-PAGE, and the proteins were transferred to a nitrocellulose membrane, and then incubated with rabbit anti-Rap1 (1:1000 dilution; Cell Signalling Technology) for immunoblotting. The levels of β-actin in the total protein lysates were also blotted for assessment of equal protein loading.

4.4.10 Measurement of Akt phosphorylation

The levels of total Akt1 and phospho-Akt1 (S473) were determined with Phospho (Ser473)/Total Akt Whole Cell Lysate Kit (Meso Scale Discovery, Rockville, MD). The levels of total Akt2, phospho-Akt2 (S474) and phospho-Akt1/2 (T308/309) were determined with Western blotting. Briefly, neutrophil lysates in RIPA buffer were mixed with 4× Laemmli buffer (Volume ratio 2:1) at 95°C for 5 min, resolved by 10% SDS-PAGE, and transferred to a nitrocellulose membrane and immunoblotted as described previously [320]. The nitrocellulose membrane was blocked with 5% BSA at room temperature for 1 h and then incubated at 4°C overnight with rabbit anti-phospho-Akt2 (S474, 1:1000 dilution; Cell Signalling Technology) or rabbit anti-phospho-Akt1/2 (T308/309, 1:1000 dilution; Bioss, Woburn, MA). After incubation with HRP-conjugated respective secondary antibodies (1:4000 dilution; Enzo Life Sciences, Cedarlane, Burlington, ON, Canada), the protein binding was detected with Clarity ECL Substrates (Bio-rad, Montreal, QC, Canada). The developed blots were washed with stripping buffer (containing in mM, glycine 200, sodium dodecyl sulfate 3.47, and 1% Tween-20; pH 2.2) for 2 h and reprobed respectively with rabbit anti-Akt2 (1:1000 dilution; Cell Signalling Technology) and mouse anti-β-actin (1:2000 dilution; Invitrogen, Burlington, ON, Canada) antibodies, followed by incubation with corresponding secondary antibodies and Clarity ECL Substrates. Image Studio Lite (Version 5.2, LI-COR Biotechnology, Lincoln, NE) was used for densitometric quantification of the detected bands.

4.4.11 Statistical analysis

Data are expressed as arithmetic means ± SEM from at least three independent experiments. Statistical analyses of the differences among groups were performed using a two-sided Student *t*-test or one-way ANOVA followed by the Holm-Sidak method, with $p<0.05$ considered to be statistically significant. Analyses were conducted using GraphPad Prism 7 (GraphPad Software, La Jolla, CA).

4.5 Results

4.5.1 Genotyping for Bam32$^{-/-}$ mice

Bam32$^{-/-}$ mice carry a Bam32 gene in which only exon 1 is replaced by a neomycin resistance cassette. Therefore, Bam32$^{-/-}$ mice used throughout this study were genotyped to verify the replacement of the exon 1. Ear tissue clips were collected from each breeding parents of Bam32$^{-/-}$ mice and were genotyped by PCR with the primers described in the *4.4 Material and methods*.

As shown in Figure 4-1, the samples from WT mice yielded a PCR product of around 450 bp, representing the presence of WT allele. By contrast, samples from all breeding parents of Bam32$^{-/-}$ mice yielded PCR products of around 800 bp, representing the replaced allele as described previously [263].

4.5.2 TNF-α-induced neutrophil recruitment in Bam32$^{-/-}$ mice

To investigate the role of Bam32 in neutrophil recruitment in mice, we first administered TNFα, a pro-inflammatory cytokine that activates endothelial cells and triggers the massive production of various chemokines, to mouse cremaster muscle *in vivo*. As shown in Figure 4-2A and 4-2B, deficiency of Bam32 significantly reduced the leukocyte rolling velocity and rolling flux even without TNFα. Moreover, treatment with TNFα over 3.5 h further decreased leukocyte rolling velocity, but still maintained the markedly differences in leukocyte rolling velocity between the two mouse strains. In spite of the decreased leukocyte rolling velocity, our results show that deficiency of Bam32 failed to change the number of adherent neutrophils or emigrated neutrophils in the mouse cremaster muscle treated with TNFα, suggesting that Bam32 may play a role in TNFα-induced leukocyte rolling velocity but not in subsequent leukocyte recruitment. However, considering that TNFα binds to its receptors on endothelial cells rather than directly stimulates circulating leukocytes, and also induces the production of many intermediary chemoattractants released from or presented on endothelial cells, we cannot completely exclude a possible role of Bam32 in leukocyte adhesion and emigration through endothelial cells simply based on this “shot-gun” approach.

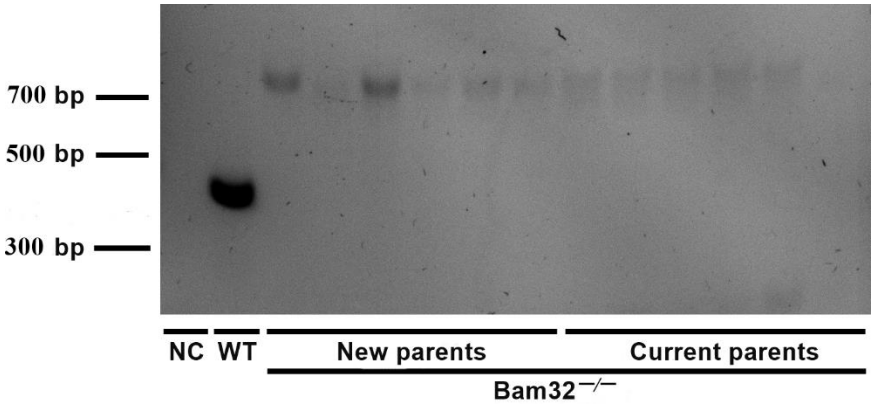

Figure 4-1. Representative Southern blot image of PCR products. NC, negative control; WT, wild-type.

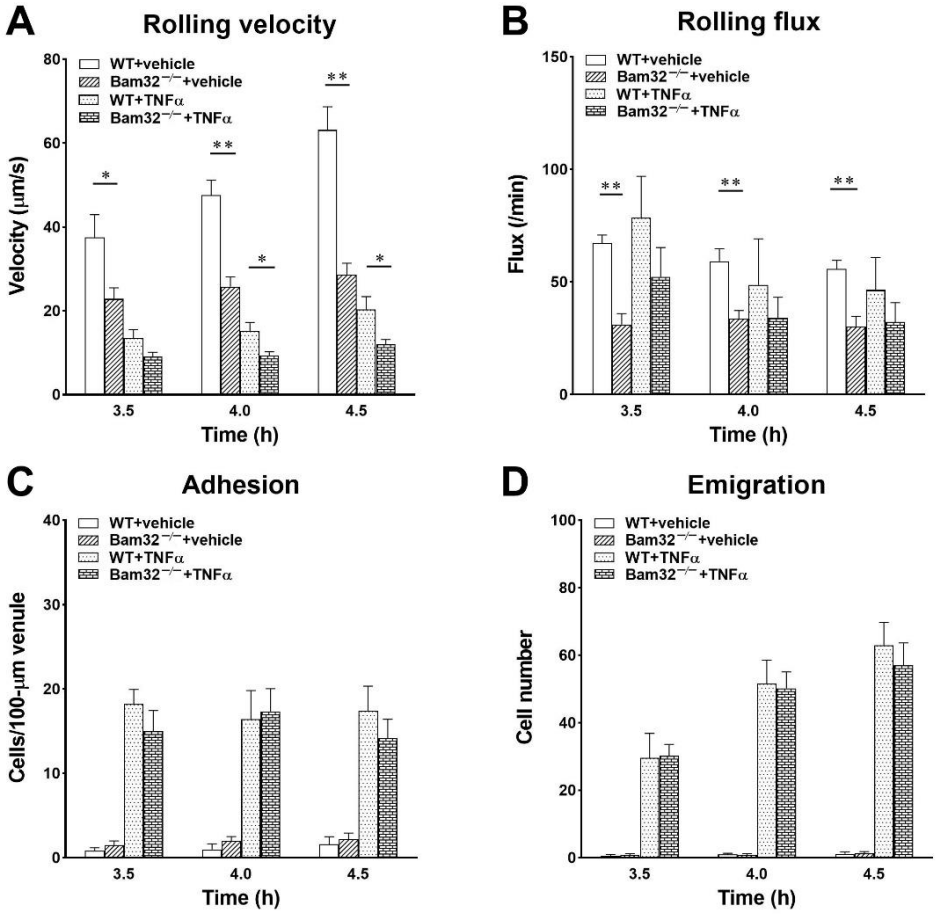

*Figure 4-2. Deficiency of Bam32 decreases TNFα-induced leukocyte rolling velocity and flux in mouse cremaster muscle. Leukocyte rolling velocity (A), rolling flux (B), the numbers of adherent neutrophils (C, cells/100-μm venule) and emigrated neutrophils (D, cells/235 × 208 μm² fields) at 3.5 h, 4 h, and 4.5 h following the intrascrotal injection with vehicle (saline) or TNFα in WT and Bam32$^{-/-}$ mice. A–D, mean ± SEM of 6 mice per group. */** indicate significant differences (*: p<0.05 and **: p<0.01) between WT and Bam32$^{-/-}$ mice.*

4.5.3 Bam32 maintains CXCL2-induced leukocyte rolling flux and rolling velocity in mouse cremaster muscle

Since TNFα-induced neutrophil recruitment is the consequence of the combined effects of multiple chemokines, to investigate the role of Bam32 in chemokine-induced neutrophil recruitment in mice, we replaced TNFα with CXCL2, a small CXC chemokine secreted by monocytes and macrophages and chemotactic for polymorphonuclear neutrophils. Considering the rapid responses induced by CXCL2 as compared to TNFα, we placed a small piece of agarose gel containing CXCL2 on the mouse cremaster muscle to allow the formation of CXCL2 gradient, instead of injecting CXCL2 intrascrotally. Our results show that a global deficiency of Bam32 failed to change leukocyte rolling velocity in the first 60 min after the placement of CXCL2-containing gel. However, it significantly decreased leukocyte rolling flux at 60 min but not at 30 min after the treatment with CXCL2. These results suggest that Bam32 may play a role in CXCL2-induced leukocyte recruitment in mouse cremaster muscle *in vivo*.

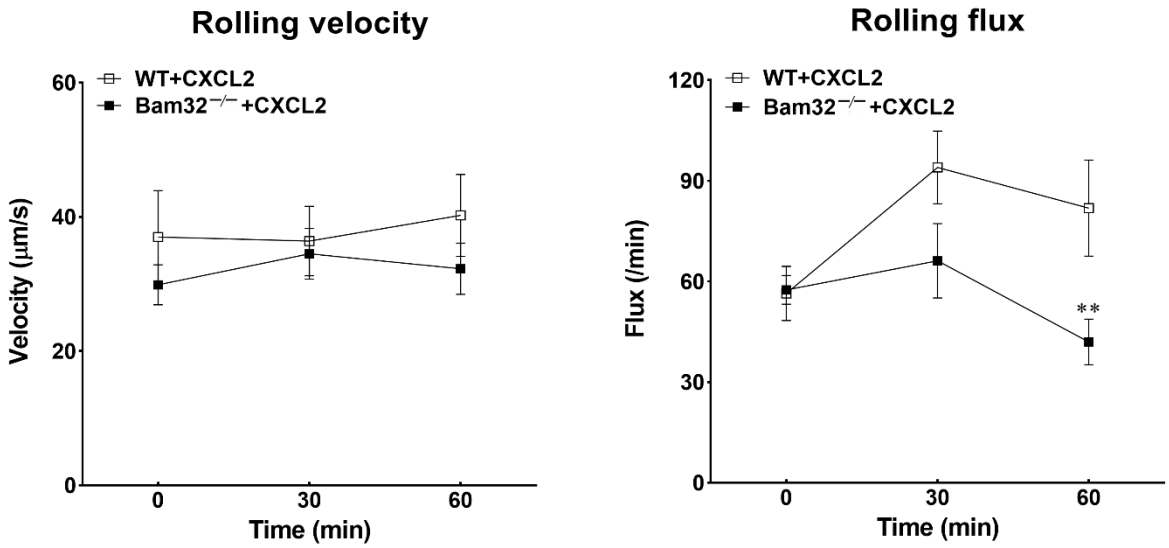

*Figure 4-3. Deficiency of Bam32 decreases CXCL2-induced leukocyte rolling flux in mouse cremaster muscle. Leukocyte rolling velocity (left) and rolling flux (right) determined at 30 min and 60 min following the placement and held of CXCL2-containing gel on cremaster muscle of WT and Bam32$^{-/-}$ mice. Mean ± SEM of 5 mice per group. ** indicates a significant difference ($p<0.01$) from WT mice.*

4.5.4 Bam32 suppresses CXCL2-induced transmigration and chemotaxis in mouse cremaster muscle

We further explored the role of Bam32 in each single step of CXCL2-induced neutrophil recruitment in mouse cremasteric post-capillary venules. As shown in Figure 4-4 and 4-5, deficiency of Bam32 significantly increased neutrophil transmigration after 60 min treatment with CXCL2 without changing neutrophil adhesion. By making and analyzing the 512× time-lapsed movie, we further examined which steps of the whole recruitment process that deficiency of Bam32 enhanced in mouse cremaster muscle. Figure 4-6 shows that deficiency of Bam32 failed to change neutrophil intraluminal crawling distance, intraluminal crawling velocity, transendothelial time and detachment time of the transmigrated neutrophils. By quantifying the numbers of total adherent, crawling and transmigrated cells, we found that the percentages of adherent cells that transmigrated and of crawling cells that transmigrated, but not the percentage of adherent cells that crawled, were significantly increased by Bam32 deficiency (Figure 4-6). Moreover, Figure 4-7 shows that, after having transmigrated into cremaster muscle tissue, the extravascular Bam32$^{-/-}$ neutrophils, as compared with WT neutrophils, demonstrated significantly increased chemotaxis distance and chemotaxis velocity, but similar migration distance, migration velocity and chemotaxis index, the last of which represents the directionality of neutrophil movement. Together, these results indicate that Bam32 suppresses CXCL2-induced neutrophil transmigration and chemotaxis in mouse cremaster muscle.

4.5.5 Bam32 suppresses CXCL2-induced neutrophil emigration in mouse peritoneum

Because neutrophils in different organs respond differently to even the same stimulus during inflammation [350], we explored whether deficiency of Bam32 enhances CXCL2-induced neutrophil recruitment into the mouse peritoneum. As shown in Figure 4-8, after 2-h treatment with CXCL2, deficiency of Bam32 significantly increased the numbers of total emigrated leukocytes as well as emigrated neutrophils. Interestingly, after 4-h treatment with CXCL2, the significant differences in the numbers of total emigrated leukocytes, emigrated neutrophils, and emigrated

macrophages between Bam32$^{-/-}$ and WT mice disappeared. Given that continuous stimulation with CXCL2 may increase the expression of adhesion molecules on the surface of emigrated cells and peritoneal cells, longer treatment with CXCL2 may still trigger more emigrated cells but reduce the "lavagibility" of those emigrated cells by strengthening their binding to the peritoneum. Meanwhile, we quantified the numbers of other emigrated leukocyte subsets in the peritoneum of both WT and Bam32$^{-/-}$ mice, but deficiency of Bam32 failed to change the number of macrophages, lymphocytes, eosinophils, and basophils in CXCL2-treated mouse peritoneum at either time points (2 h and 4 h). These results indicate that Bam32 suppresses CXCL2-induced neutrophil recruitment in peritonitis.

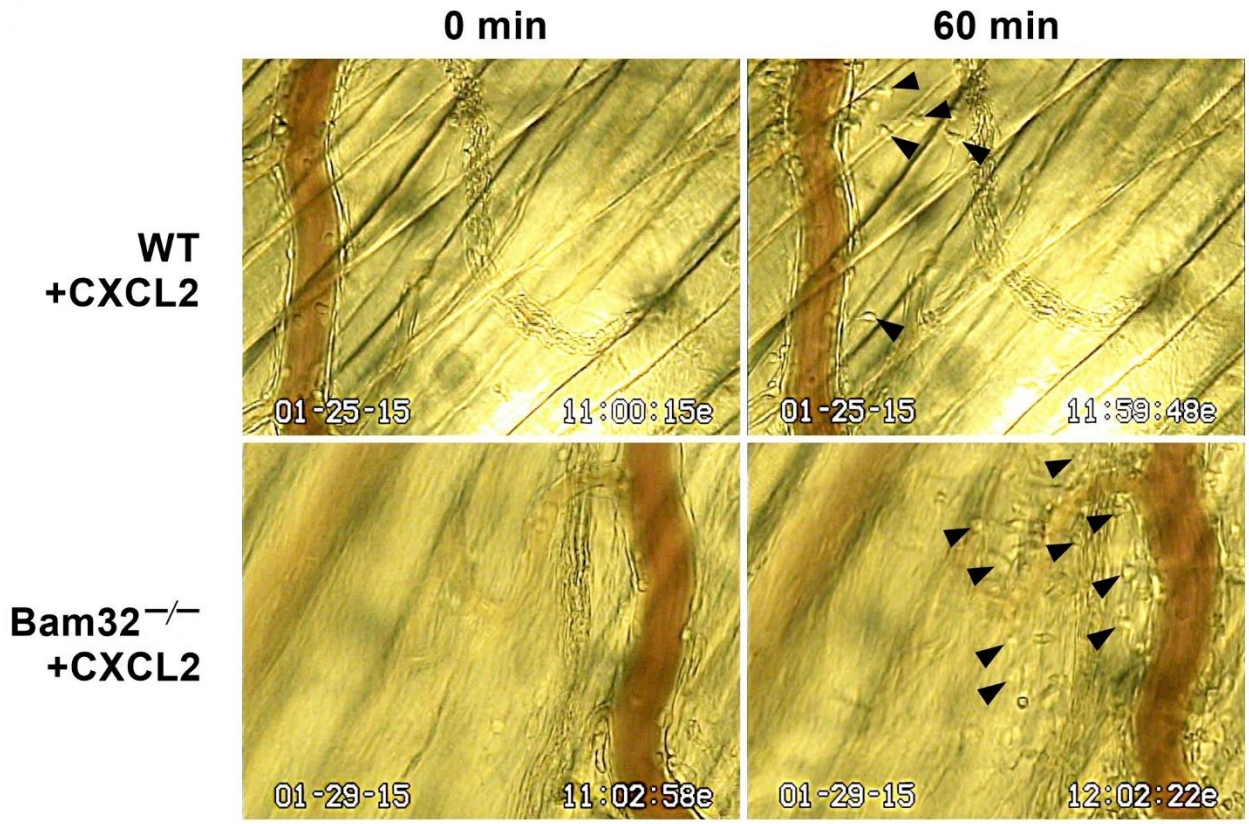

Figure 4-4. CXCL2-induced neutrophil recruitment in mouse cremaster muscle (micrograph). Representative micrographs from intravital video microscopy showing a postcapillary venule and the surrounding tissue of cremaster muscle with transmigrated neutrophils (arrowhead) before (0 min) and at 60 min following the placement of the CXCL2-containing gel (out of view, 350-µm distant from the venule) in WT mice and Bam32$^{-/-}$ mice (Magnification: 100×).

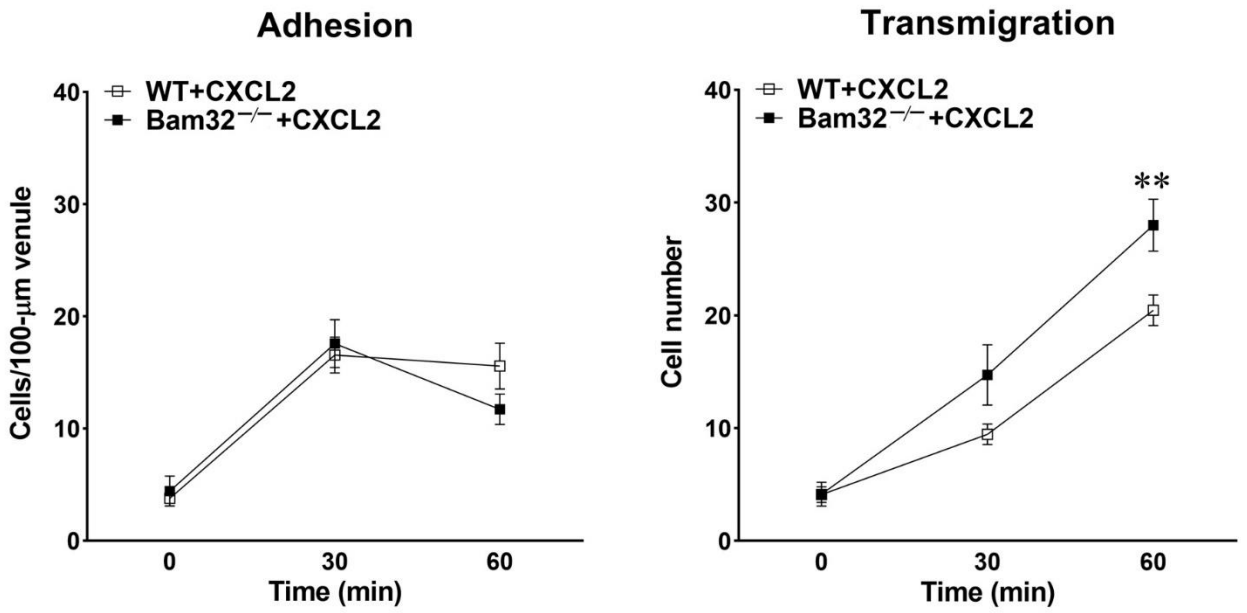

*Figure 4-5. Deficiency of Bam32 increases CXCL2-induced neutrophil transmigration in mouse cremaster muscle. The numbers of adherent neutrophils (cells/100-µm length of venule) and emigrated neutrophils (cells/235 × 208 µm^2 fields) at 30 min and 60 min following the placement of CXCL2-containing gel on cremaster muscle of WT mice and Bam32$^{-/-}$ mice. Mean ± SEM of 7 mice per group. ** indicates significant difference ($p<0.01$) from WT mice.*

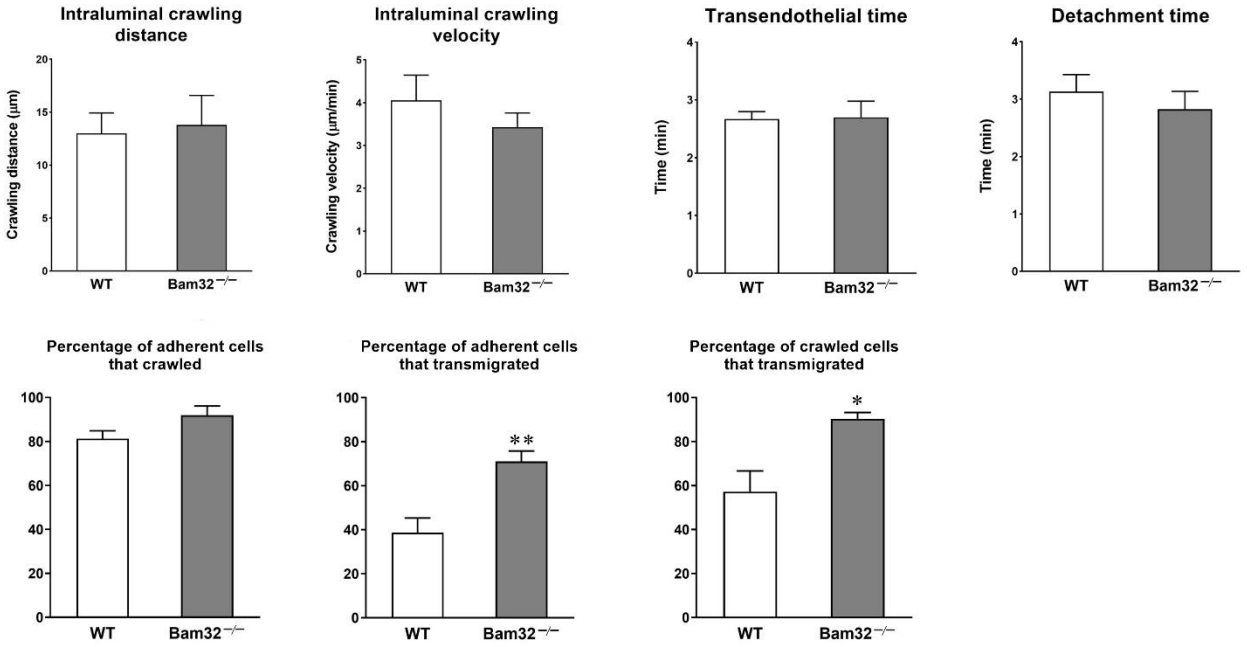

*Figure 4-6. Effect of Bam32 deficiency on CXCL2-induced neutrophil transendothelial migration in mouse cremaster muscle. The crawling distance (μm), crawling velocity (μm/min), transendothelial time (min), detachment time (min), the percentage of adherent cells that crawl on the luminal surface of the endothelium, the percentage of adhesion cells that transmigrate across the endothelium, and the percentage of crawling cells that transmigrate across the endothelium during 60-min video recording following the placement of CXCL2-containing gel in WT mice and Bam32$^{-/-}$ mice (averaged from >50 cells). Mean ± SEM of 5 mice per group. * and ** indicate significant differences ($p<0.05$ and $p<0.01$) from WT mice.*

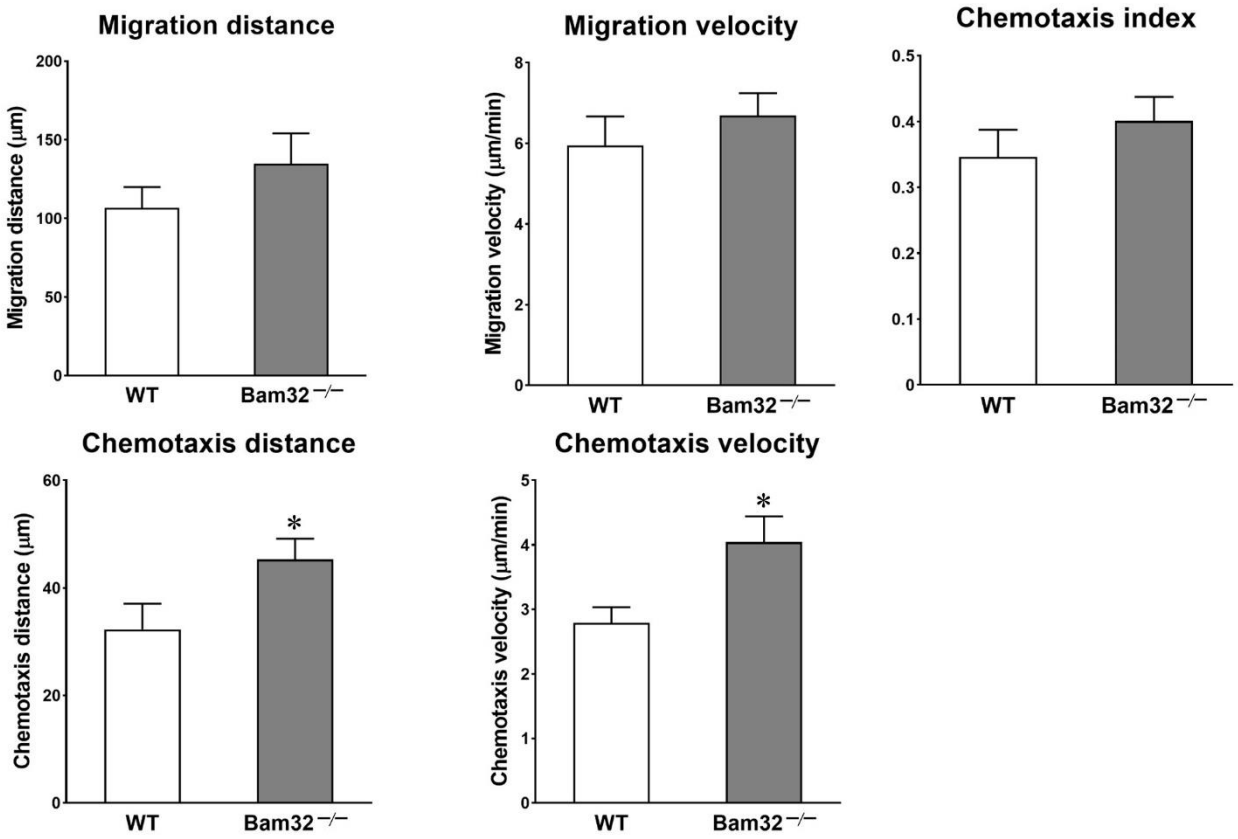

*Figure 4-7. Deficiency of Bam32 increases CXCL2-induced neutrophil chemotaxis distance and velocity in mouse cremaster muscle. The migration distance (μm), chemotaxis distance (μm), migration velocity (μm/min), chemotaxis velocity (μm/min), and chemotaxis index of neutrophil chemotaxis in extravascular tissue during 60 min following the placement and held of CXCL2-containing gel on cremaster muscle of WT mice and Bam32$^{-/-}$ mice (averaged from >80 cells). Mean ± SEM of 7 mice per group. * indicates significant difference ($p<0.05$) from WT mice.*

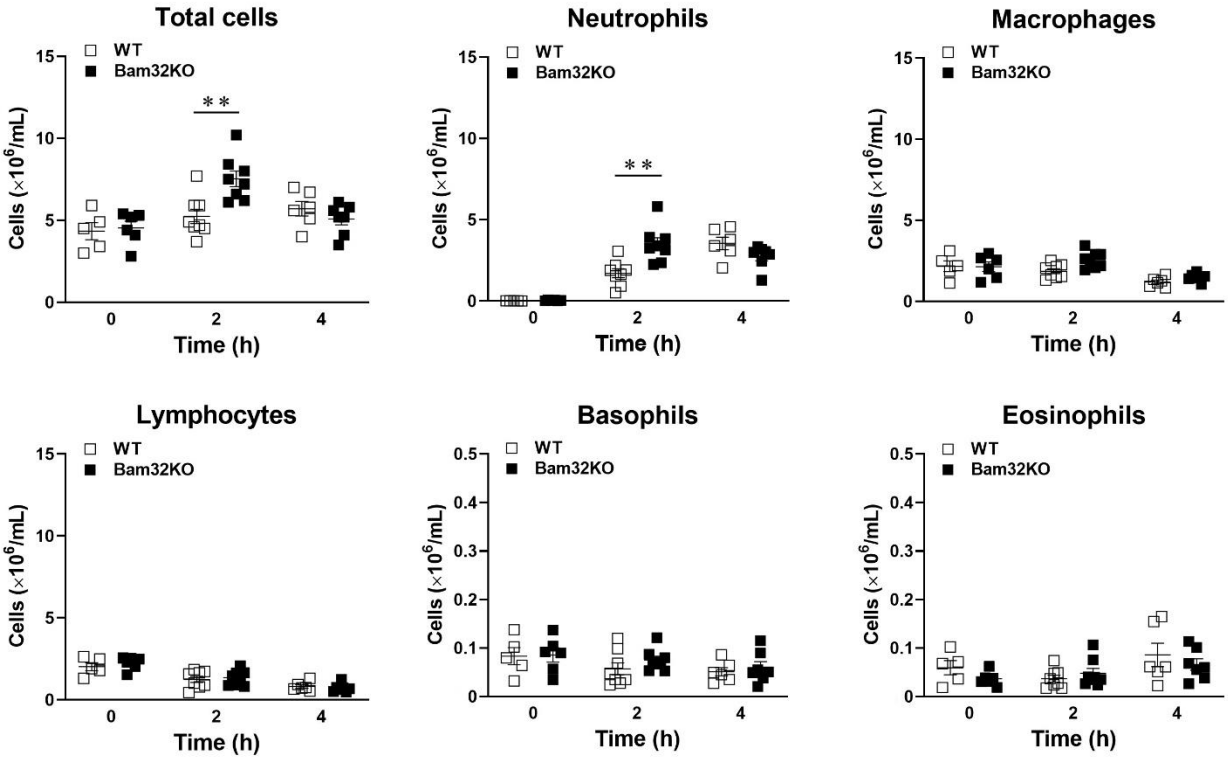

*Figure 4-8. Deficiency of Bam32 increases CXCL2-induced neutrophil emigration in mouse peritoneum. The number of total emigrated leukocytes, neutrophils, macrophages, lymphocytes, basophils, and eosinophils after intraperitoneal injection with CXCL2 (0.5 µg/25 g body weight). Mean ± SEM of 5–8 mice per group. ** indicates significant differences ($p<0.01$) from WT mice.*

4.5.6 Oyster glycogen-induced emigration of neutrophils and other leukocyte subsets in the peritoneum of Bam32$^{-/-}$ mice

In addition to chemokine CXCL2, we also determined the numbers of total leukocytes and other leukocyte subsets in mouse peritoneum treated with glycogen from oyster, a branched polymer of glucose for inducing inflammation and promoting extravasation of granulocytes and neutrophils through TLR2 in animal models after intraperitoneal injection [351]. Unlike the trends in mouse peritonitis after 2-h or 4-h treatments with CXCL2, our results show that deficiency of Bam32 decreased the numbers of total leukocytes and neutrophils without changing the numbers of other leukocyte subsets in mouse peritoneum after 4-h treatment with oyster glycogen (Figure 4-9). These results, together with the results of CXCL2-induced mouse peritonitis, suggest that the roles of Bam32 in neutrophil recruitment in mouse peritoneum may vary among the activation of different neutrophil surface receptors.

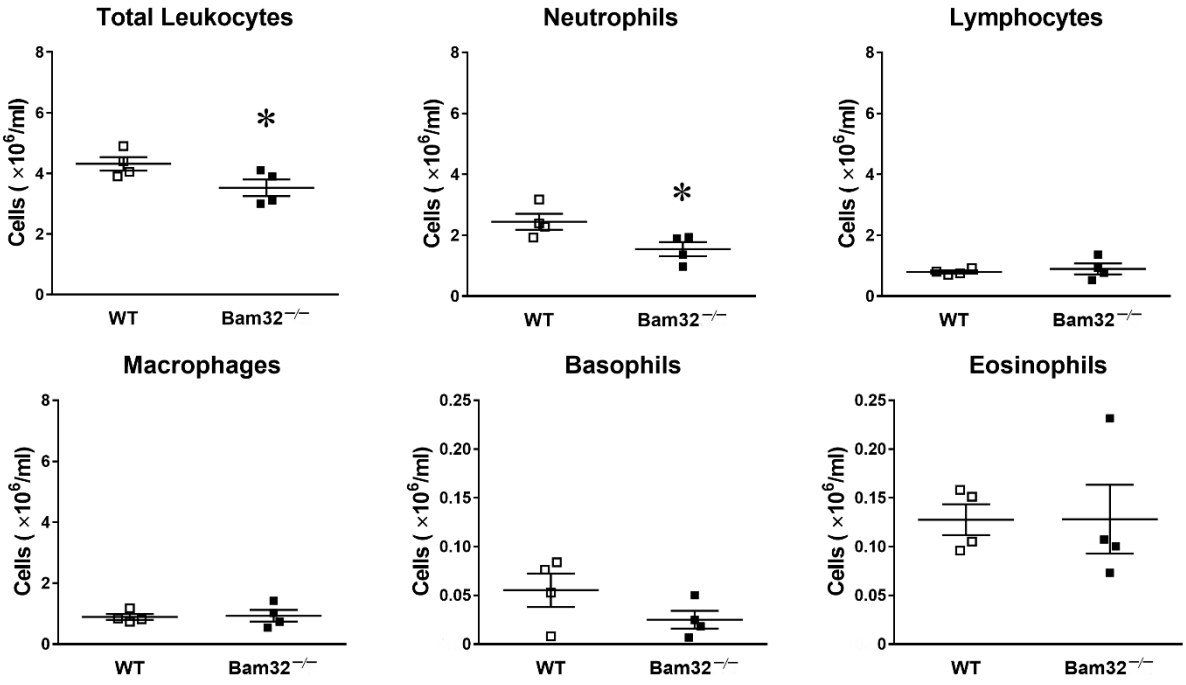

*Figure 4-9. Deficiency of Bam32 decreases oyster glycogen-induced neutrophil emigration in mouse peritoneum. The numbers of total leukocytes, neutrophils, lymphocytes, macrophages, basophils, and eosinophils at 4 h after intraperitoneal injection with glycogen from oyster (10 mg/25 g body weight). Mean ± SEM of 4 mice per group. * indicates significant differences ($p<0.05$) from WT mice.*

4.5.7 Bam32 suppresses CXCL2-induced neutrophil chemotaxis *in vitro*

The neutrophil-endothelium interaction was reported to play a dominant role in neutrophil extravasation during acute inflammation by many previous studies [352]. To investigate the role of Bam32 only in neutrophils without involving endothelium, we determined CXCL2-induced migration distance, migration velocity, chemotaxis distance, chemotaxis velocity, and chemotaxis index of bone marrow-derived primary neutrophils and of oyster glycogen pre-stimulated, peritoneum neutrophils *in vitro*. As shown in Figure 4-10, in bone marrow-derived neutrophils, deficiency of Bam32 significantly increased neutrophil chemotaxis distance and chemotaxis velocity *in vitro*, an observation that was consistent with our results obtained in CXCL2-treated mouse cremaster muscle. Interestingly, in pre-stimulated, peritoneum neutrophils, deficiency of Bam32 increased chemotaxis distance and migration velocity but not chemotaxis velocity. This minor discrepancy might be due to the activation of Toll-like receptors by oyster glycogen during neutrophil stimulation in the peritoneum in addition to the activation of receptors for CXCL2 [353]. Together, these results provide further evidence that Bam32 suppresses CXCL2-induced neutrophil migration and chemotaxis *in vitro*.

4.5.8 Rac1 activation is upregulated whereas Rap1 activation is downregulated in Bam32$^{-/-}$ neutrophils

In view of the modulatory effects of Bam32 on the small GTPases required for cytoskeletal rearrangement after activation of CXC chemokine receptor in B cells [266], we investigated CXCL2-induced activation of Rac1 and Rap1 by measuring GTP-bound Rac1 and Rap1 in bone marrow-derived WT and Bam32$^{-/-}$ neutrophils. As shown in Figure 4-11A, the deficiency of Bam32 significantly increased the levels of activated GTP-bound Rac1 in neutrophils with or without CXCL2. Figure 4-11B shows that deficiency of Bam32 resulted in suppression of GTP-bound Rap1 activation with CXCL2 stimulation but had no apparent effect on changing the total Rap1 expression. Given that Rac1 activation is required in Rap1 activation in neutrophils [354], we then explored whether inhibition of Rac1 activation reduced the activation of Rap1 in CXCL2-

stimulated neutrophils by applying specific Rac1 inhibitor NSC23766. As shown in Figure 4-12, inhibition of Rac1 activation suppressed Rap1 activation in WT mice, without changing the expression of total Rap1. Our data indicate that the presence of Bam32 and the activation of Rac1 are both required for Rap1 activation in mouse neutrophils.

4.5.9 Pharmacological inhibition of Rac1 and Rap1 modulates CXCL2-induced neutrophil chemotaxis *in vitro*

To investigate whether modulation of Rac1 or Rap1 activation changes CXCL2-induced neutrophil chemotaxis, we incubated fresh bone marrow-derived neutrophils with NSC23766 (Rac1 inhibitor) or GGTI298 (Rap1 inhibitor) respectively for 30 min prior to and throughout 1-h CXCL2 stimulation *in vitro*. As depicted in Figure 4-13, inhibition of Rac1 significantly suppressed the chemotaxis distance and chemotaxis velocity of CXCL2-treated Bam32$^{-/-}$ neutrophils. This inhibition also reduced the migration velocity without substantially changing the migration distance of CXCL2-treated Bam32$^{-/-}$ neutrophils. Moreover, inhibition of Rac1 significantly suppressed the directionality of chemotaxis (chemotaxis index) of CXCL2-treated Bam32$^{-/-}$ neutrophils. By contrast, inhibition of Rap1 activation failed to enhance the chemotaxis of CXCL2-treated Bam32$^{-/-}$ neutrophils, but significantly increased chemotaxis distance, chemotaxis velocity, migration velocity, and chemotaxis index of CXCL2-treated WT neutrophils. Moreover, significant differences in chemotaxis distance and chemotaxis index were noted between WT and Bam32$^{-/-}$ neutrophils treated by NSC23766. Together, our results suggest that modulating the activation of Rac1 or Rap1 regulates downstream CXCL2-induced neutrophil chemotaxis *in vitro* and Rac1 remains active in maintaining CXCL2-induced neutrophil migration and chemotaxis responses when Bam32 is absent.

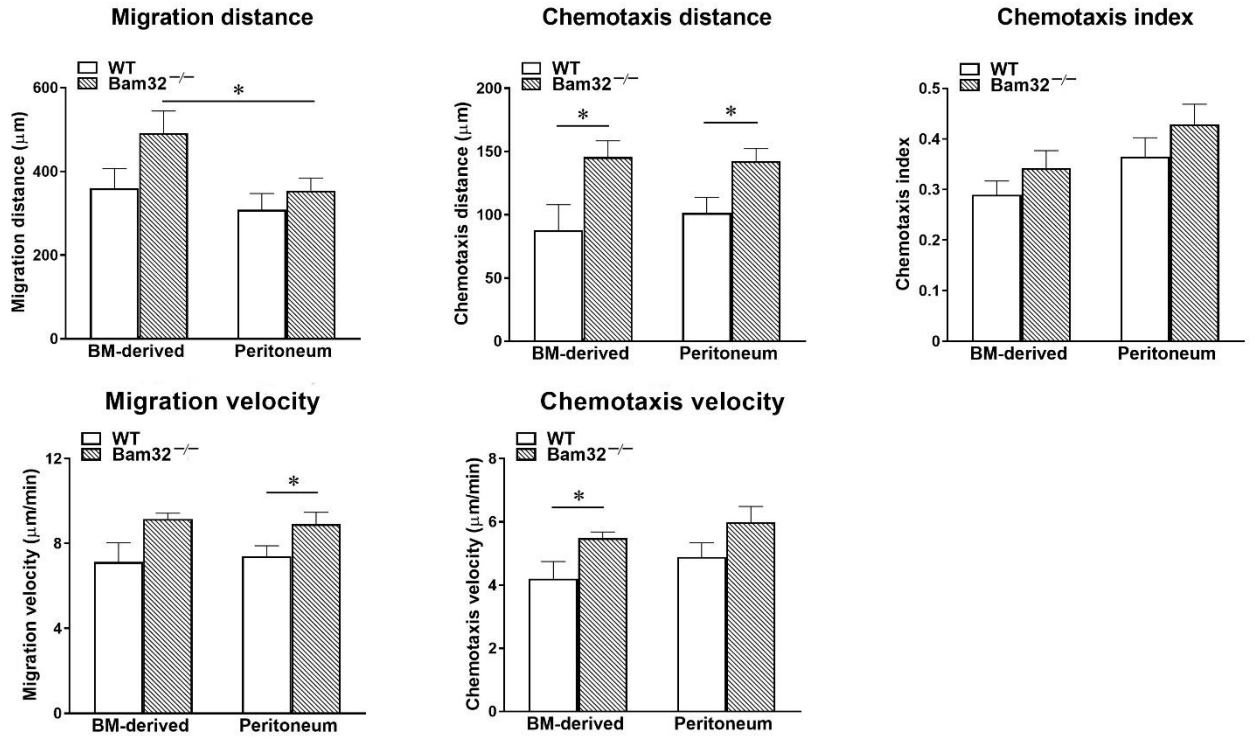

*Figure 4-10. Deficiency of Bam32 increases CXCL2-induced chemotaxis of bone marrow-derived neutrophils and pre-stimulated peritoneum neutrophils in vitro. The migration distance (μm), chemotaxis distance (μm), chemotaxis index, migration velocity (μm/min) and chemotaxis velocity (μm/min) for 60 min in 2D chemotaxis μ-slide (averaged from >50 cells). Mean ± SEM of 4 mice per group. * indicates significant differences ($p<0.05$) between the two groups. BM, bone marrow.*

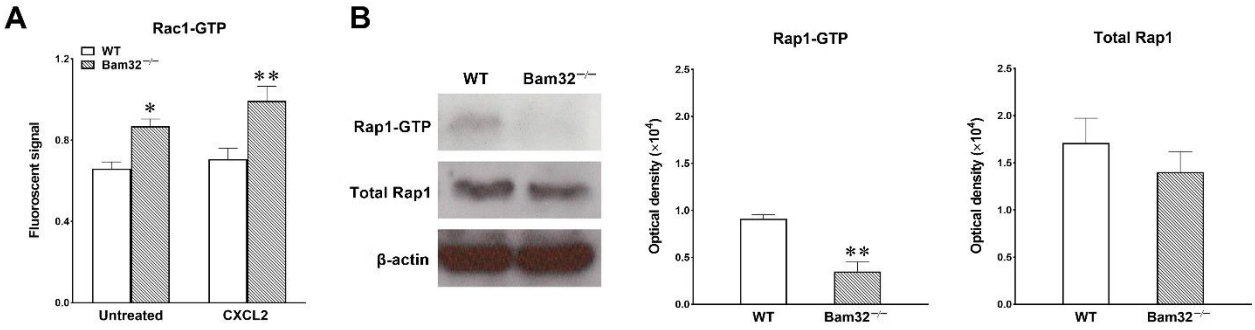

*Figure 4-11. Deficiency of Bam32 changes CXCL2-induced Rac1 and Rap1 activation in neutrophils. (A) Levels of Rac1-GTP in bone marrow-derived neutrophils from WT and Bam32$^{-/-}$ mice without CXCL2 (Untreated) and at 2 min after stimulation with CXCL2 (0.1 μM). (B) Representative original Western blots and the levels of Rap1-GTP and total Rap1 determined in bone marrow-derived neutrophils from WT and Bam32$^{-/-}$ mice at 2 min after stimulation with CXCL2 (0.1 μM). A–C, mean ± SEM of 3 samples per group. Each sample was collected from two mice of the same strain. The Rac1 GLISA assay was performed duplicately in 96-well plate. * and ** indicate significant differences ($p<0.05$ and $p<0.01$) from WT neutrophils.*

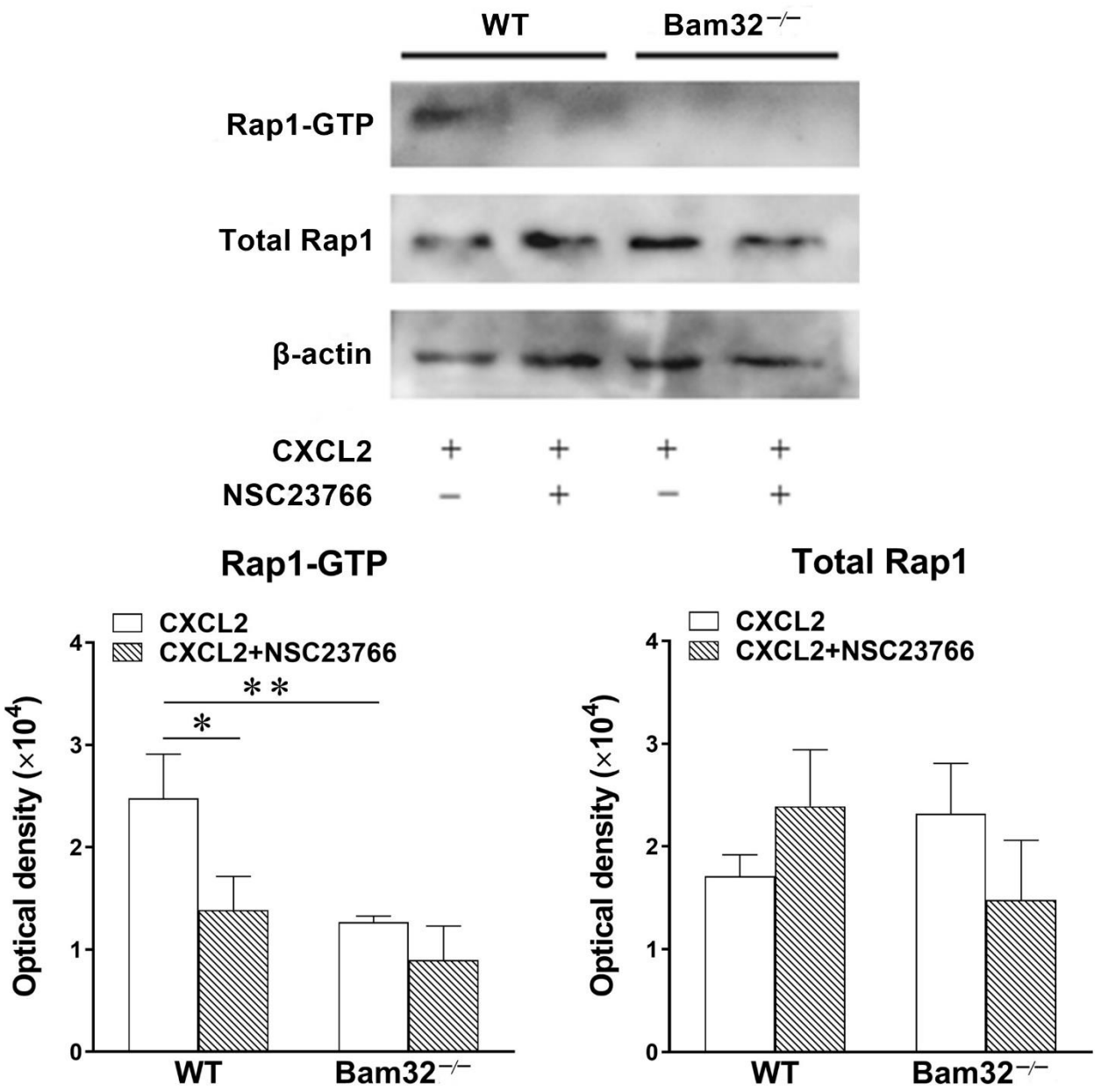

*Figure 4-12. Pharmacological inhibition of Rac1 activation impairs Rap1 activation in WT neutrophils. Representative original Western blot and the levels of Rap1-GTP and total Rap1 determined in bone marrow-derived neutrophils from WT and Bam32$^{-/-}$ mice at 2 min after stimulation with CXCL2 (0.1 μM) without or with pre-treatment with NSC23766 (50 μM) for 30 min and throughout 2-min CXCL2 stimulation. A–C, mean ± SEM of 3 samples per group. Each sample was collected from two mice of the same strain. * and ** indicate significant differences ($p<0.05$ and $p<0.01$) from WT neutrophils without NSC23766.*

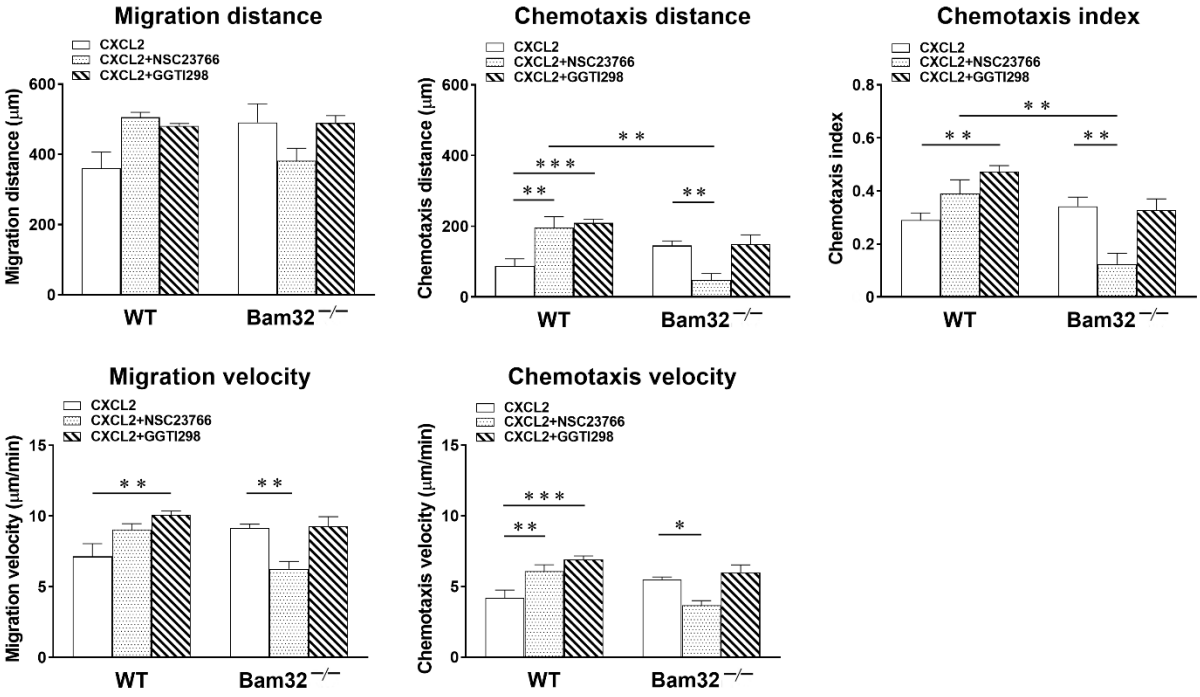

*Figure 4-13. Pharmacological inhibition of Rac1 and Rap1 on CXCL2-induced neutrophil chemotaxis in vitro. The migration distance (μm), chemotaxis distance (μm), chemotaxis index, migration velocity (μm/min) and chemotaxis velocity (μm/min) for 60 min without or with pre-treatment with Rac1 inhibitor NSC23766 (50 μM) or Rap1 inhibitor GGTI298 (10 μM) in 2D chemotaxis μ-slide (averaged from >50 cells). Mean ± SEM of 4 mice per group. *, ** and *** indicate significant differences ($p<0.05$, $p<0.01$ and $p<0.001$, respectively) between the two groups.*

4.5.10 Rap1 activation suppresses CXCL2-induced Akt1/2 phosphorylation in neutrophils

Because activation of Rap1 was revealed to suppress the phosphorylation of Akt which is the key modulator downstream of PI3K to regulate chemoattractant-induced recruitment in mouse neutrophils [355], we, therefore, explored whether the deficiency of Bam32 which impaired Rap1 activation changed CXCL2-induced phosphorylation of Akt. As shown in Figure 4-14 and 4-15, deficiency of Bam32 had no effect on levels of total Akt1 and Akt2 or CXCL2-induced phosphorylation of Akt at Ser473 and Ser474 but enhanced CXCL2-induced phosphorylation of Akt1/2 at Thr308/309 in neutrophils. Moreover, inhibition of Rap1 activation by GGTI298 further increased phospho-Akt1/2 at site Thr308/309 in Bam32$^{-/-}$ neutrophils treated with CXCL2. Thus, our results suggest that Bam32-dependent Rap1 activation suppresses Akt phosphorylation induced by CXCL2 in mouse neutrophils. Moreover, Bam32-independent Rap1 activation also has a suppressive effect but maybe inferior to Bam32-dependent Rap1 activation on the phosphorylation of Akt at Thr308/309.

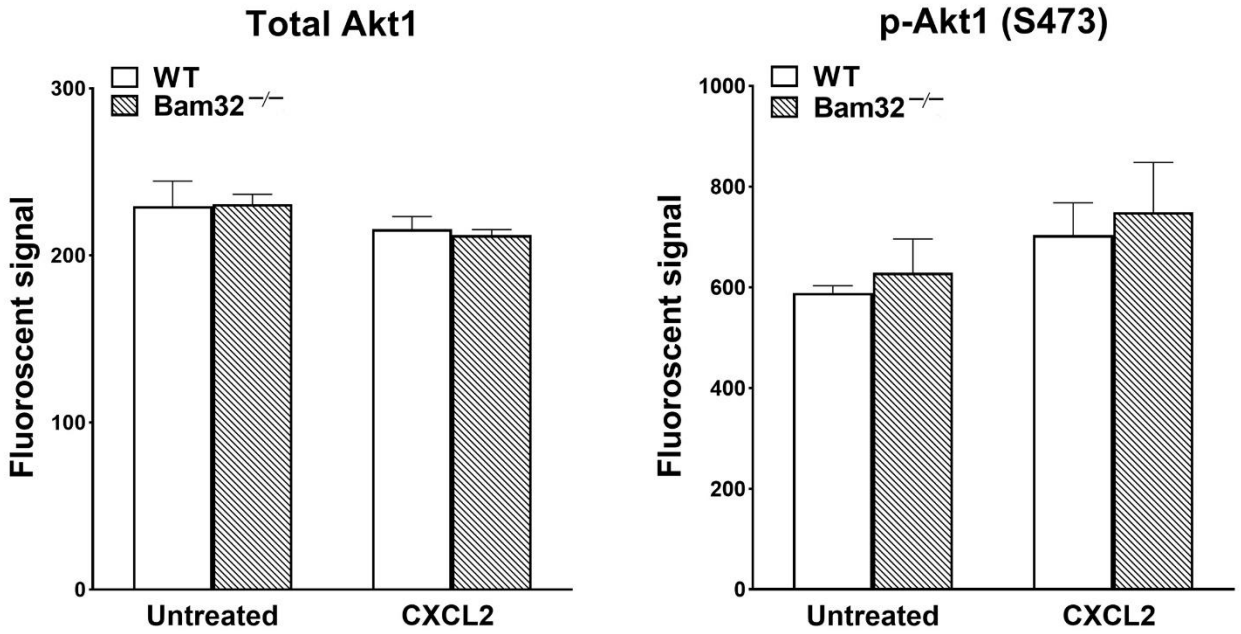

Figure 4-14. Deficiency of Bam32 has no effect on CXCL2-induced phosphorylation of Akt1 at Ser473 in neutrophils. The levels of total Akt1 and phospho-Akt1 at Ser473 determined in bone marrow-derived neutrophils from WT and Bam32$^{-/-}$ mice without or at 5 min after stimulation with CXCL2 (0.1 μM). mean ± SEM of 4 samples per group. Each sample was collected from two mice of the same strain.

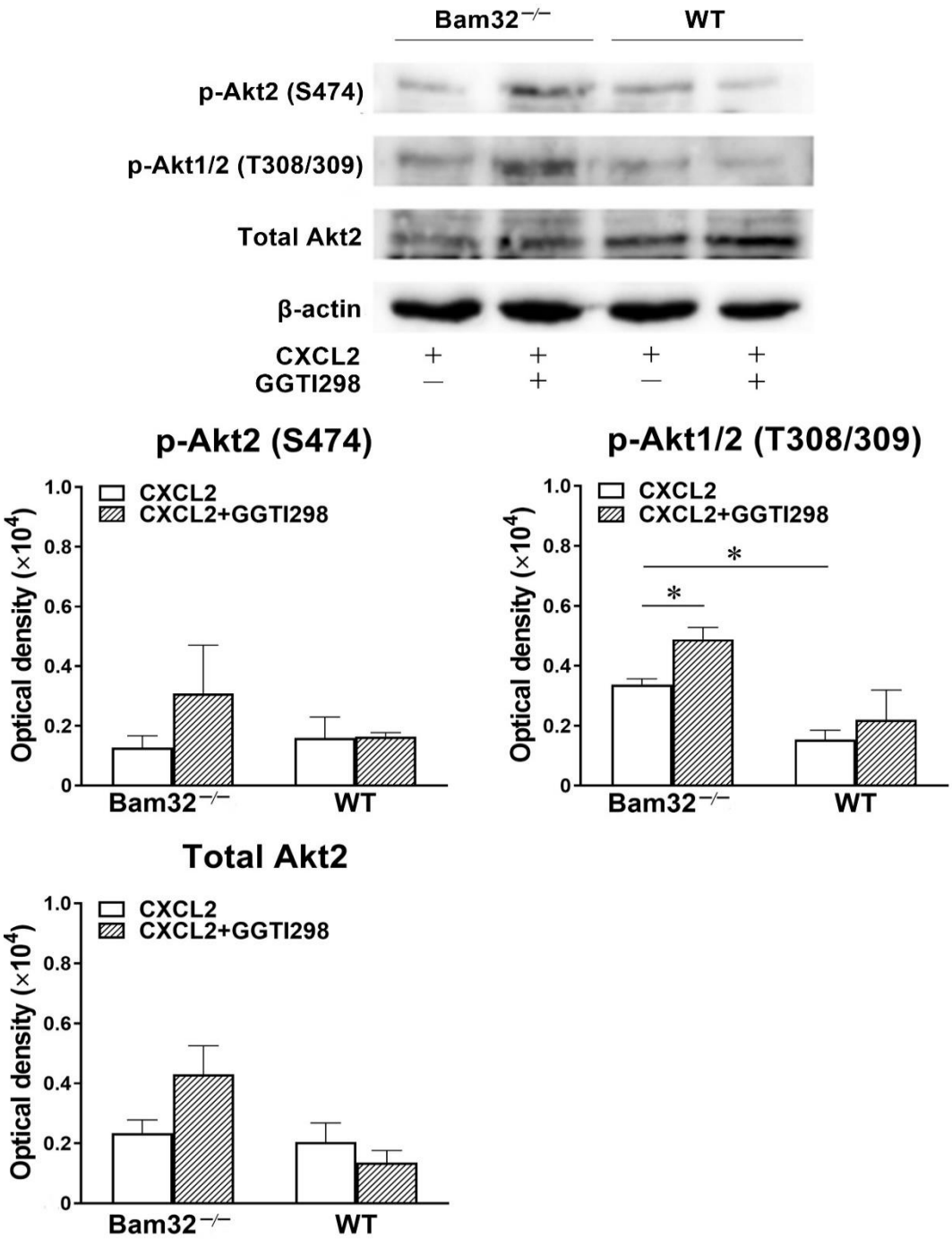

*Figure 4-15. Deficiency of Bam32 increases CXCL2-induced phosphorylation of Akt1/2 at Thr308/309 in neutrophils. Representative original Western blot and the levels of phospho-Akt2 at S474, phospho-Akt1/2 at Thr308/309 and total Akt2 determined in bone marrow-derived neutrophils from WT and Bam32$^{-/-}$ mice at 5 min after stimulation with CXCL2 (0.1 μM) without or with treatment with GGTI298 (10 μM) for 30 min prior to and throughout 5-min CXCL2 stimulation. Mean ± SEM of 3 samples per group. Each sample was collected from two mice of the same strain. * indicates significant differences between the two groups ($p<0.05$).*

4.5.11 Pharmacological inhibition of Akt suppresses CXCL2-induced neutrophil recruitment and chemotaxis in cremaster muscle of Bam32$^{-/-}$ mice

Here, we sought to elucidate if inhibition of Akt attenuated CXCL2-induced enhancement in neutrophil recruitment resulted from a deficiency of Bam32 in mouse cremaster muscle. SH-5 (pan-Akt inhibitor, 1 μM) was superfused on the cremaster muscle for 30 min prior to and throughout 1-h stimulation with CXCL2. As shown in Figure 4-16, SH-5 failed to change the numbers of adherent and transmigrated neutrophils in WT mice, but significantly decreased the numbers of adherent and transmigrated neutrophils in Bam32$^{-/-}$ mice at both 30 min and 60 min. Interestingly, after application of SH-5, the numbers of adherent and transmigrated neutrophils in Bam32$^{-/-}$ was significantly lower than those in WT mice, suggesting that the importance of Akt in neutrophil recruitment is substantially increased when Bam32 is absent.

By further analyzing the time-lapsed movie, we found that inhibition of Akt with SH-5 significantly decreased migration distance and chemotaxis distance without changing the directionality of neutrophil chemotaxis (chemotaxis index), migration velocity or chemotaxis velocity in Bam32$^{-/-}$ mice (Figure 4-17). These data indicate that the activation of Akt, which is suppressed by Bam32-dependent Rap1 activation, is required in CXCL2-induced neutrophil recruitment in mice.

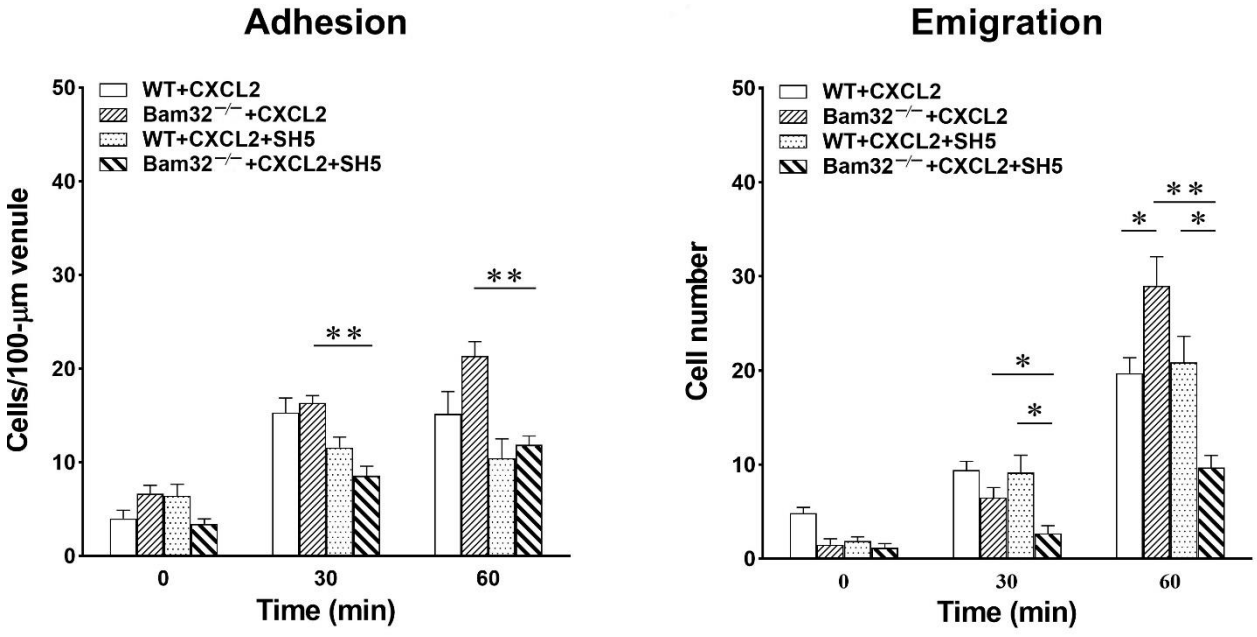

*Figure 4-16. Pharmacological inhibition of Akt decreases CXCL2-induced neutrophil adhesion and emigration in mouse cremaster muscle. The number of adherent neutrophils (cells/100-μm length of venule) and emigrated neutrophils (cells/235 × 208 μm^2 field) at 30 min and 60 min following the placement and held of CXCL2-containing gel on cremaster muscle of WT and Bam32$^{-/-}$ mice without or with superfusion with SH-5 (1 μM) for 30 min prior to and throughout 1-h CXCL2 stimulation. Mean ± SEM of 4 mice per group. * and ** indicate significant differences ($p<0.05$ and $p<0.01$).*

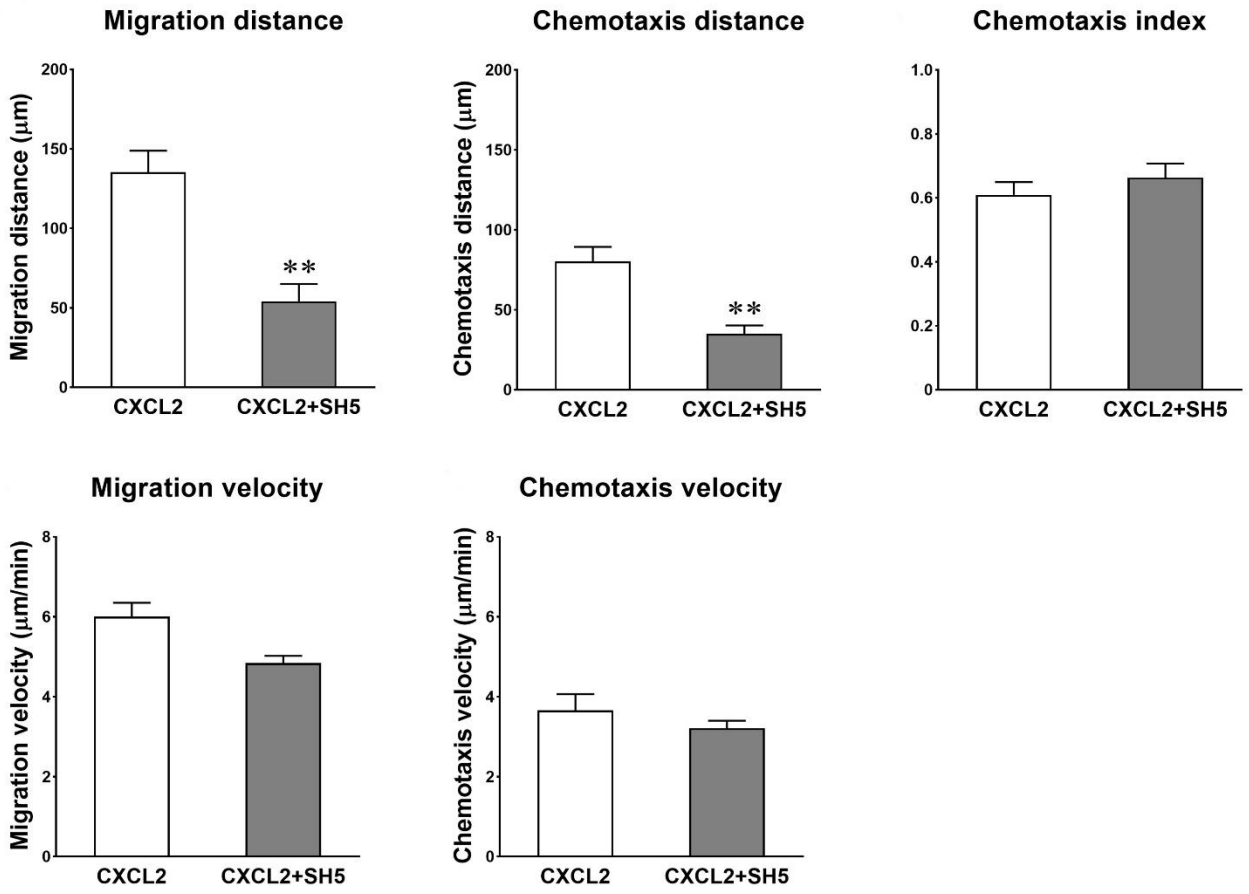

*Figure 4-17. Pharmacological inhibition of Akt decreases CXCL2-induced neutrophil chemotaxis in mouse cremaster muscle. The migration distance, chemotaxis distance, chemotaxis index, migration velocity, and chemotaxis velocity of neutrophil chemotaxis in extravascular tissue during 60 min following the placement and held of CXCL2-containing gel on cremaster muscle of Bam32$^{-/-}$ mice (averaged from >50 cells) without or with superfusion with SH-5 (1 μM) for 30 min prior to and throughout 1-h CXCL2 stimulation. Mean ± SEM of 4 mice per group. ** indicates significant differences ($p<0.01$) between CXCL2-treated Bam32$^{-/-}$ mice with and without SH-5 (C–G).*

4.6 Discussion

Mounting research evidence demonstrates that G protein-coupled PI3K, its activation, and its downstream adaptor proteins are implicated in orchestrating neutrophil recruitment during acute inflammation [220,356]. Our study is the first to unveil a hitherto unknown suppressive role of Bam32, one of the PI3K downstream adaptors originally discovered in B cells, in neutrophil transmigration and chemotaxis induced by CXC chemokine *in vivo* and *in vitro*. We show that deficiency of Bam32 enhanced CXCL2-induced neutrophil transmigration in mouse cremaster muscle and mouse peritoneum at early time points and reveal that deficiency of Bam32 in neutrophils increased CXCL2-induced neutrophil chemotaxis distance and velocity without involving endothelial cells *in vitro*. The underlying mechanism of the suppressive role of Bam32 in neutrophil recruitment was through modulating Rap1 activation, which was evidenced by the measurements of neutrophil Rap1 activation, migration, and chemotaxis after application of GGTI298 (specific Rap1 inhibitor) to bone marrow-derived neutrophils from both mouse strains. Moreover, Rap1-mediated Akt signaling was involved in these neutrophil responses, because the inhibition of Rap1 increased CXCL2-induced Akt1/2 phosphorylation at T308/309, the activation of which played a pivotal role of downstream neutrophil recruitment in mouse cremaster muscle.

A supportive role of Bam32 was reported in modulating BCR-induced, F-actin-mediated cytoskeletal remodeling in B lymphocytes [266]. However, our observation points to a suppressive role of Bam32 in regulating chemokine-induced neutrophil recruitment, because deficiency of Bam32 substantially increased CXCL2-induced neutrophil transmigration in mouse cremaster muscle. This augment in neutrophil transmigration may be due to the increased initiation of transmigration as evidenced by the increased percentages of adhesion cells that transmigrated and of crawling cells that transmigrated through a Bam32-dependent mechanism. Although neutrophil adhesion is regarded as one of the important prerequisites for neutrophil transmigration [21,357,358], our data show no difference in neutrophil adhesion between WT and Bam32$^{-/-}$ mice. It is probably because 50 percent decline in leukocyte rolling flux at 60 min in Bam32$^{-/-}$ mice may not be sufficient to significantly increase neutrophil adhesion. Therefore, our results exclude the possibility that Bam32-dependent changes in neutrophil transmigration are caused by the changes

in rolling or adhesion. In extravascular tissue, deficiency of Bam32 increased neutrophil chemotaxis distance and velocity without changing migration distance, migration velocity and chemotaxis index. This suggests that Bam32 may be involved in the neutrophil chemotactic movement without influencing other neutrophil activities such as chemotactic directionality in mouse cremaster muscle.

Although the classical paradigm of neutrophil recruitment is largely established in mouse cremaster muscle and mesentery by the use of intravital microscopy [359], recruitment mechanisms may deviate in different organs. This deviation is owing to the organ-specific vasculature, perivascular cells, and organ-specific tissue-resident cell [360,361]. In our study, the suppressive role of Bam32 in mouse neutrophil recruitment was verified in CXCL2-induced neutrophil emigration in mouse peritoneum. Deficiency of Bam32 increased emigrated neutrophil number as early as 2 h after intraperitoneal injection of CXCL2, a result that was in line with our observation of neutrophil recruitment within 1-h CXCL2 treatment in mouse cremaster muscle. Intriguingly, in CXCL2-induced mouse peritonitis, the difference resulted from a deficiency of Bam32 in the number of transmigrated neutrophils was diminished at 4 h after intraperitoneal injection of CXCL2, indicating that Bam32-dependent regulation of neutrophil emigration may only be responsive to CXCL2 at early time points.

The neutrophil-endothelial adhesive interaction is a sophisticated process that regulates subsequent neutrophil extravasation from the microvasculature [352,358,362,363]. Neutrophil firm adhesion mediated by β2-integrin is the fundamental step that triggers the intrinsic signaling for neutrophil transmigration [83], which includes two distinct transendothelial pathways (paracellular and transcellular) [364]. In paracellular transendothelial migration, the intrinsic signaling and its adaptor proteins in endothelial cells activate the disassembly of VE-cadherin complex at cell-cell junctions, which allows adherent neutrophils to squeeze through the gap between endothelial cells [122,365]. In transcellular transendothelial migration, endothelial cells form nanoscale adhesion platforms that contribute to the formation of a ring- or cup-like macromolecular structure at the base of adherent neutrophils [366,367]. This structure, called “endothelial dome”, in part dependent

on leukocyte-specific protein 1 (LSP1) [31], facilitates the membrane fusion at the endothelial-neutrophil interface and promotes neutrophil transendothelial migration [32]. Here, we scrutinized the role of Bam32 in neutrophils by determining CXCL2-induced extravascular neutrophil chemotaxis in isolated neutrophils from the two mouse strains *in vitro*. Our data show that, consistent with our recruitment results *in vivo*, deficiency of Bam32 enhanced CXCL2-induced neutrophil chemotaxis *in vitro*. Moreover, although neutrophil desensitization may decrease the amplitude of neutrophil responses [368], the emigrated neutrophils pre-stimulated by oyster glycogen in our study demonstrated the same trend as the bone marrow-derived unstimulated neutrophils in chemotaxis parameters. The minimal effect of desensitization that may be involved in our models might be due to the different receptors and signaling pathways activated by oyster glycogen and CXCL2 used respectively in this study.

Rap1, a member of Ras family small GTPases, has a regulatory role in neutrophil recruitment. Rap1a is required for E-selectin-dependent slow rolling in leukocytes after activation of CalDAG-GEFI and p38 MAPK signal pathways [295]. Moreover, Rap1b suppresses neutrophil transendothelial migration and chemotaxis by inhibiting Akt in the PI3K signaling pathway [296]. Our results strongly indicate that Bam32 is required for Rap1 activation, which suppresses CXCL2-induced neutrophil chemotaxis. Interestingly, as shown in our study and elsewhere [354], Bam32 is not the only prerequisite for Rap1 activation. The activation of Rac1, a Rho family small GTPase that is regulated by Bam32 in B lymphocytes through a tyrosine phosphorylation-dependent mechanism [266], is equally important for CXCL2-induced Rap1 activation in mouse neutrophils. Nevertheless, the mechanisms of how Bam32 modulates the activation of Rap1 directly or through regulating Rac1 activation warrant future studies.

The integrin $\alpha_M\beta_2$-dependent Akt signaling in neutrophil recruitment is regulated by Rap1b, as Rap1b deficiency enhances Akt phosphorylation through increasing $\alpha_M\beta_2$ cross-linking [296]. Akt has three different isoforms—Akt1, Akt2, and Akt3, among which Akt1 and Akt2 are involved in leukocyte migration, and Akt3 controls the growth and development of mice and humans [369–371]. In previous studies, Akt1 in endothelial cells was found to increase B-cell migration through

activating STAT5 [372] and to enhance neutrophil migration via elevating microvascular permeability [298]. By contrast, Akt2 performs its role in neutrophils by controlling neutrophil degranulation and migration. This identified mechanism challenges the existing paradigm that all Akt proteins have similar functions in neutrophil recruitment [355]. Our study provides evidence that deficiency of Bam32 increases Akt phosphorylation at site Thr308/309 without influencing other phosphorylation sites or the total levels of Akt1 and Akt2. In this study, we found that the Bam32-dependent modulation in CXCL2-induced Akt phosphorylation was mediated by Rap1 because inhibition of Rap1 by GGTI298 increased CXCL2-induced Akt phosphorylation at Thr308/309 in Bam32$^{-/-}$ neutrophils. Moreover, this Bam32-dependent suppressive role of Rap1 sequentially modulates CXCL2-induced Akt-mediated neutrophil recruitment, which is evidenced by the suppression of neutrophil adhesion and emigration in Bam32$^{-/-}$ mice but not in WT mice after application of pan-Akt inhibitor SH-5. In this regard, our study reveals a mechanism that Bam32-dependent Rap1 activation suppresses neutrophil recruitment through inhibiting PI3K/Akt signaling. This suppressive role of Bam32 in neutrophils is in line with a previous study revealing that Bam32-deficient mast cells have enhanced IgE-induced activation, which is owing to reduced activation of SHIP, the PI3K pathway suppressor [261].

One of the limitations in this study is that we cannot exclude the possibility that Bam32 may regulate neutrophil recruitment via Rac1 because Rac1 is also reported to play a profound role in neutrophil chemotaxis [373]. Different from the role of Rac2 in neutrophil superoxide production [374] and in integrin-mediated spreading and actin assembly [277], Rac1 participates in the formation of leading-edge and uropod events *via* regulating Rho and myosin activation in both human and mouse neutrophils [279]. Moreover, Rac1 also modulates the gradient detection to chemokines in neutrophils [277,375]. Our study reveals that deficiency of Bam32 increases neutrophil basal and CXCL2-induced Rac1 activity, accompanied by increased neutrophil chemotaxis *in vitro*. Pharmacological inhibition of Rac1 activation by NSC23766 abolishes the enhancement in chemotaxis of Bam32$^{-/-}$ neutrophils, suggesting that Bam32 may regulate neutrophil chemotaxis through modulating Rac1 activation. However, inhibition of Rac1 activation

in WT mice failed to decrease but increased the chemotaxis distance and velocity, an observation that was inconsistent with previous studies. The inconsistency may be due to the stimulus-specific mechanisms and the complex interactions among small GTPases downstream of Bam32. The exact mechanism involved in this inconsistency may require exploration in the future.

In summary, our data show an unreported suppressive role of Bam32 in regulating chemokine-induced mouse neutrophil transmigration and chemotaxis both *in vivo* and *in vitro*. This Bam32-dependent suppression may be a functional braking mechanism during neutrophil recruitment that is mediated by Rap1, which acts as an inhibitory small GTPase through suppressing Akt phosphorylation in neutrophils. Therefore, our study provides mechanistic insight into the role of PI3K adaptor protein Bam32 in neutrophil recruitment during chemokine-induced acute inflammation.

CHAPTER FIVE

BAM32/DAPP1-DEPENDENT NEUTROPHIL REACTIVE OXYGEN SPECIES IN WKYMVM-INDUCED MICROVASCULAR HYPERPERMEABILITY

5.1 Preface

In this chapter, we unveil a novel role of Bam32 in regulating WKYMVm-induced hyperpermeability in mouse post-capillary venules through Bam32-dependent ERK1/2-related ROS generation in neutrophils. This study, for the first time, reveals the role of Bam32 in the interaction between innate immune cells and the microvasculature. Meanwhile, this study reveals that Bam32, albeit an adaptor for PI3K signaling pathway, may also function in MAPK signaling pathway in neutrophils.

Data presented in this chapter has been included in a research paper by Li Hao, Aaron J. Marshall, and Lixin Liu entitled "Bam32/DAPP1-dependent neutrophil reactive oxygen species in WKYMVm-induced microvascular hyperpermeability" in the *Frontiers in Immunology* 2020 May 27. The contents of this chapter have been adapted from the manuscript. In this study, Li Hao conducted all the experiments, performed data analysis, drafted the manuscript, and participated in the study design, data interpretation and manuscript revision.

5.2 Abstract

B cell adaptor molecular of 32 kDa (Bam32), also known as dual adapter for phosphotyrosine and 3-phosphoinositides 1 (DAPP1), has been implicated in regulating lymphocyte proliferation and recruitment during inflammation. However, its role in neutrophils during inflammation remains unknown. Using intravital microscopy, we examined the role of Bam32 in formyl peptide WKYMVm-induced permeability changes in post-capillary venules and assessed simultaneously neutrophil adhesion and emigration in cremaster muscles of Bam32-deficient ($Bam32^{-/-}$) and wild-type (WT) mice. We observed significantly reduced WKYMVm-induced microvascular hyperpermeability accompanied by markedly decreased neutrophil emigration only in $Bam32^{-/-}$ mice. The Bam32-specific decrease in WKYMVm-induced hyperpermeability was neutrophil-dependent as this was verified in chimeric mice transplanted with bone marrow from $Bam32^{-/-}$ mice. We discovered that Bam32 was critical for WKYMVm-induced intracellular and extracellular production of reactive oxygen species (ROS) in neutrophils. Pharmacological scavenging of ROS eliminated the differences in WKYMVm-induced hyperpermeability between $Bam32^{-/-}$ and WT mice. In addition, deficiency of Bam32 decreased WKYMVm-induced ERK1/2 but not p38 or JNK phosphorylation in neutrophils. Inhibition of ERK1/2 signaling cascade suppressed WKYMVm-induced ROS generation in WT neutrophils and microvascular hyperpermeability in WT mice. In conclusion, our study reveals that Bam32-dependent, ERK1/2-involving ROS generation in neutrophils is critical in formyl peptide-induced microvascular hyperpermeability during neutrophil recruitment.

5.3 Introduction

B cell adaptor molecule of 32 kDa (Bam32), also known as a dual adaptor for phosphotyrosine and 3-phosphoinositides 1 (DAPP1), is an intracellular protein widely expressed in various subsets of leukocytes, including B lymphocytes, T lymphocytes, dendritic cells and mast cells [257]. As an adaptor protein downstream of phosphoinositide 3-kinase (PI3K), Bam32 promotes B cell chemotaxis through regulating Rac1-mediated cytoskeletal rearrangement, actin remodeling, membrane ruffling and lamellipodia formation [266]. It also integrates PI3K and Src kinase signals to promote germinal center progression in B cells as well as B cell adhesion and communication with T cells [269,271]. Beyond the PI3K signaling pathway, the role of Bam32 in the mitogen-activated protein kinase (MAPK) pathway was also reported in the activation of B cells [376]. Bam32 is required in p38 MAPK-mediated B cell proliferation and T cell-independent Ag responses of B cells [260,377]. In addition, Bam32 facilitates protein tyrosine kinase-mediated B cell Ag receptor internalization [378].

In other adaptive immune cell types, Bam32 is required for TCR-mediated cytokine production, proliferation and actin-mediated spreading of $CD4^+$ T cells [272]. Bam32 in dendritic cells promotes antigen-presenting functions and increases MHC class I-induced $CD8^+$ T cell activation [262]. Beyond its functions in the adaptive immune cells, Bam32 constrains granule release through reducing FcεRI-induced calcium flux in mast cells [261]. Although the roles of Bam32 have been established mostly in the adaptive immunity and only one innate cell population (mast cells) during the past two decades, its role in many other innate immune cell types including neutrophils remains unclear.

Trp-Lys-Tyr-Met-Val-D-Met-NH_2 (WKYMVm) is a synthetic agonist that mimics bacterial formyl peptide f-Met-Leu-Phe (fMLP) and activates leukocytes *via* formyl peptide receptors (FPR) [306,307]. WKYMVm preferentially stimulates FPR2 and is 1000-fold stronger than natural bacterial peptide fMLP in ROS production from mouse leukocytes [306,379]. WKYMVm also triggers the chemotactic migration of leukocytes as an end-target chemoattractant [317,380]. In addition to its effects on the immune system, WKYMVm functions in large vessels of the

cardiovascular system by reversing LPS-induced vascular hyporeactivity to phenylephrine in the mouse aorta by decreasing the production of nitric oxide [308]. In Chapter 3, I showed that PI3Kγ, an upstream regulator of Bam32 signaling in neutrophils, regulates WKYMVm-induced microvascular hyperpermeability. Other than that, the effect of WKYMVm on the crosstalk between the immune system and microvasculature is largely unknown.

In this study, we used intravital microscopy and fluorescence imaging *in vivo* to explore the role of Bam32 in WKYMVm-induced hyperpermeability in mouse post-capillary venules. We determined the effect of Bam32-dependent, ERK1/2-involving mechanism of ROS production in neutrophils on the change of microvascular barrier functions during neutrophil recruitment.

5.4 Material and methods

5.4.1 Animals

Bam32-deficient ($Bam32^{-/-}$) mice were generated by Dr. Michel Nussenzweig [263] on the C57BL/6 background and transferred to the University of Saskatchewan. Male mice between 6 and 12-week-old were used in the experiments along with age-matched male C57BL/6N mice (wild-type, WT) purchased from Charles River Canada (Saint-Constant, QC, Canada). This study was carried out with the protocol (#20070028) approved by the University Committee on Animal Care and Supply at the University of Saskatchewan and following the standards of the Canadian Council on Animal Care. All efforts were made to reduce animal suffering and all surgeries were performed under deep ketamine-xylazine anesthesia.

5.4.2 Measurement of microvascular permeability, neutrophil adhesion, and neutrophil emigration

Jugular vein cannulation was performed on mice that were anesthetized after an intraperitoneal (i.p.) injection of a cocktail of ketamine (200 mg/kg, Roger, Montreal, QC, Canada) and xylazine (10 mg/kg, Bayer, Toronto, ON, Canada). The mouse cremaster muscle was surgically exposed as previously described [312,313], and superfused with 37°C-warmed bicarbonate-buffered physiological saline (pH 7.4; containing in mM, NaCl 133.9, KCl 4.7, $MgSO_4$ 1.2 and $NaHCO_3$ 20.0; all reagents purchased from Fisher Scientific, Toronto, ON, Canada). The bright-field and fluorescent intravital microscopy was performed under an upright BX61WI Olympus microscope (Olympus, Tokyo, Japan) with an LUCPLFLN 20× objective lens. The permeability index, calculated as the ratio of extravascular fluorescence intensity (FI) to the adjacent intravascular FI of the observed cremasteric venule, was measured as previously described [314–316]. Briefly, FITC-labelled bovine serum albumin (BSA, 25 mg/kg, Sigma-Aldrich, Oakville, ON, Canada) was infused into the mouse circulation through the jugular vein 5 min prior to 1-h superfusion of exposed cremaster muscle with WKYMVm (0.1 μM, Phoenix Pharmaceutical, Burlingame, CA) [317] or the control saline. Fluorescence intensity (excitation wavelength: 495

nm; emission wavelength: 525 nm) was detected using a monochrome deep-cooled CCD camera (Retiga SRV, QImaging, Surrey, BC, Canada) and quantified by the MAG biosystems software (MetaMorph®, Molecular Devices Inc., PA). Fluorescence images were taken on the cremasteric venule every 5 min during superfusion with WKYMVm or the saline. The numbers of adherent and emigrated neutrophils were determined under bright-field microscopy prior to (0 min) and after 60-min superfusion with WKYMVm or the saline as described [312]. The emigrated neutrophils were defined as the neutrophils emigrated to the extravascular tissue from the venule, excluding the neutrophils that attached on the tunica media. The number of emigrated neutrophils in intravital microscopy was counted in the field of 443×286 μm^2 (two full screens on the monitor) adjacent to the recorded venule. All the above reagents were prepared in 37°C-warmed bicarbonate-buffered saline before superfusion. Where indicated, BVD-523 (Ulixertinib, 5 μM, Selleckchem, Houston, TX) or PD98059 (50 μM, Tocris, Minneapolis, MN) was pre-superfused on the cremaster muscle for 30 min prior to and throughout 60-min treatment with WKYMVm [381].

5.4.3 Harvest of neutrophils from mouse bone marrow

Murine neutrophils were freshly isolated from bone marrows of WT and Bam32$^{-/-}$ mice. The femur and tibia were dissected immediately after each mouse was sacrificed, and the marrow cavity was flushed with ice-cold calcium- and magnesium-free phosphate-buffered saline (PBS). The bone marrow cells enriched in flushing fluid were separated by three-density (72%, 64% and 52%) Percoll (GE Healthcare, Baie d'Urfe, QC, Canada) gradient centrifugation at 1060 *g* for 30 min with a previously established method, yielding over 85% morphologically mature neutrophils [319,320].

5.4.4 Bone marrow transplantation

To scrutinize the cell types where Bam32 played a role, we generated four different types of chimeric mice by transplantation of bone marrow from 5-week-old WT mice into 5-week-old WT and Bam32$^{-/-}$ recipient mice (designated as WT→WT and WT→Bam32$^{-/-}$, respectively) and of

bone marrow from 5-week-old Bam32$^{-/-}$ mice into 5-week-old WT and Bam32$^{-/-}$ recipient mice (designated as Bam32$^{-/-}$→WT and Bam32$^{-/-}$→Bam32$^{-/-}$, respectively). All recipients received two doses of X-ray irradiation (500 cGy/dose) with a 3-h interval, followed by injection of freshly prepared bone marrow cells (6–8 × 10^6 cells) from either one of the two strains of donors through mouse tail veins. Thereafter enrofloxacin (200 mg/L), freshly prepared in sterile drinking water, and sterile food were provided *ad libitum* to the chimeric mice over the following 2 weeks. The mice were housed in sterile cages for 6 weeks after bone marrow transplantation to allow the reconstitution of full immunity before experimental use.

5.4.5 Determination of intracellular ROS generation

Freshly isolated mouse neutrophils from bone marrow were incubated in 37°C-warmed Krebs-Ringer phosphate glucose solution (containing in mM, NaCl 145, Na_2HPO_4 5.7, KCl 4.86, $CaCl_2$ 0.54, $MgSO_4$ 1.22, and glucose 5.5) with 2, 7-dichlorofluorescein diacetate (DCFDA, 20 μM, Sigma-Aldrich) with gentle agitation at 37°C for 30 min, with or without the inhibitors diphenyleneiodonium (DPI, 40 μM, Sigma-Aldrich) [323,382], BVD-523 (5 μM), or PD98059 (50 μM), where indicated. DCFDA (2',7'–dichlorofluorescin diacetate) is a cell-permeable fluorogenic dye that measures hydroxyl, peroxyl, and other ROS within the cell. This dye, after entering the cells, is deacetylated by cellular esterases to a non-fluorescent compound, which will later be oxidized by ROS generated within the cells and form a highly fluorescent compound DCF. Neutrophils were washed thereafter and resuspended in 37°C DCFDA-free, inhibitor-containing Krebs-Ringer phosphate glucose solution. Fluorescent intensity was quantified with Fluoroskan Ascent Microplate Fluorometer (Thermo Fisher Scientific) upon and every 10 min after stimulation with WKYMVm (0.1 μM) or Phorbol 12-myristate 13-acetate (PMA, 0.2 μM, Sigma) [383].

5.4.6 Determination of extracellular hydrogen peroxide generation

Bone marrow-derived mouse neutrophils were freshly isolated as described above and suspended in 37°C-warmed Krebs-Ringer phosphate glucose solution. An Amplex Red Hydrogen

Peroxide assay kit (Thermo Fisher Scientific) was used to detect the production of extracellular hydrogen peroxide from neutrophils stimulated with WKYMVm (0.1 μM). The Amplex Red reagent (10-acetyl-3,7-dihydroxyphenoxazine) in combination with horseradish peroxidase (HRP) can react with the hydrogen peroxide released by cells in the medium, and produce the red fluorescent product resorufin that can be detected by fluorescence spectroscopy. All working solutions were prepared following the instructions provided by the manufacturer. Aliquots of neutrophils (1.0×10^6/mL, 0.05 mL) were mixed with the working solution (volume ratio 1:1) in black 96-well plates upon and every 10 min after stimulation. Fluorescent signals were captured by Fluoroskan Ascent Microplate Fluorometer (Thermo Fisher Scientific).

5.4.7 Rac1 activation assay

Fresh bone marrow-derived mouse neutrophils were prepared as described above and suspended in 37°C serum-free Hyclone RPMI 1640 (GE Healthcare). Colorimetric Rac1 G-LISA Activation Assay Kit (Cytoskeleton, Inc., Denver, CO) was used to quantitatively determine GTP-bound Rac1 after stimulation of neutrophils in suspension with WKYMVm (0.1 μM) at 37°C. All steps of lysing neutrophils and extracting GTP-bound Rac1 were performed strictly following the instruction provided by the manufacturer. Protein concentrations were determined using the Precision Red Advanced Protein Assay that came with the kit. Fluorescent signal of each sample on 96-well plates was captured by Fluoroskan Ascent Microplate Fluorometer (Thermo Fisher).

5.4.8 Inhibition of ROS generation in vivo

Mice were prepared for intravital microscopy and post-capillary venule permeability was determined in the cremaster muscle as described above. The NADPH oxidase inhibitor DPI (40 μM) and the hydrogen peroxide metabolizing enzyme catalase (200 units/mL, Sigma-Aldrich) [384] were prepared separately in 37°C-warmed bicarbonate-buffered saline. DMSO was used as the vehicle for DPI, whereas the inactive catalase used as a negative control was prepared by boiling the catalase solution in a water bath for 10 min. The cremaster muscle was pre-superfused with DPI or catalase for 30 min prior to and throughout the 60-min superfusion of WKYMVm.

Fluorescence images were taken when, and every 5 min after, WKYMVm was applied and permeability indices were determined at each time point as described above.

5.4.9 Western blotting

The levels of total p38, ERK1/2, SAPK/JNK MAPK, and their phosphorylated forms were determined using Western blotting. Briefly, freshly prepared bone marrow-derived mouse neutrophils were lysed in RIPA buffer, followed by mixing with 4× Laemmli buffer (volume ratio 2:1) at 95°C for 5 min. The mixtures were resolved by 10% SDS-PAGE and transferred to a nitrocellulose membrane and immunoblotted as previously described [320]. The nitrocellulose membrane was blocked with 5% BSA at room temperature for 1 h and then incubated at 4°C overnight with rabbit anti-phospho-p38, rabbit anti-phospho-ERK1/2, rabbit anti-phospho-SAPK/JNK (all three anti-phospho antibodies were used at 1:1000 dilution; Cell Signalling Technology, Whitby, ON, Canada). After incubation with HRP-conjugated goat anti-rabbit secondary antibodies (1:4000 dilution; Enzo, Burlington, ON, Canada), the blots were developed with Clarity ECL Substrates (Bio-rad, Montreal, QC, Canada). Thereafter, the developed blots were washed by stripping buffer (containing glycine 200 mM, sodium dodecyl sulfate 3.47 mM, and 1% Tween 20; pH 2.2) for 5 h and reprobed respectively with rabbit anti-p38 (1:1000 dilution; Cell Signalling Technology), rabbit anti-ERK1/2 (1:1000 dilution; Cell Signalling Technology), rabbit anti-SAPK/JNK (1:1000 dilution; Cell Signalling Technology) or mouse anti-β-actin (1:2000 dilution; Invitrogen, Burlington, ON, Canada) antibodies, followed by incubation with corresponding secondary antibodies and Clarity ECL Substrates. The Image Studio Lite (Version 5.2, LI-COR Biotechnology, Lincoln, NE) was used for densitometric quantification of the detected bands.

5.4.10 Statistical analysis

Data are expressed as arithmetic means ± SEM from at least three independent experiments. Analyses of statistical differences among groups were performed using a two-tailed Student *t*-test or one-way ANOVA followed by the Holm-Sidak *post hoc* analysis by using GraphPad Prism 7 (GraphPad Software, La Jolla, CA). $P<0.05$ was considered to be statistically significant.

5.5 Results

5.5.1 Deficiency of Bam32 decreases WKYMVm-induced microvascular hyperpermeability

To explore the role of Bam32 in murine post-capillary venule barrier functions and neutrophil recruitment, we utilized FPR agonist WKYMVm to induce microvascular leakage in WT and Bam32$^{-/-}$ mice. Vascular permeability changes over time were assessed using intravital microscopy (Figure 5-1). The baseline levels of microvascular leakage showed no significant difference between WT and Bam32$^{-/-}$ mice during 60-min superfusion with the control saline (Figure 5-2A). After superfusion with WKYMVm, the microvascular leakage in WT mice was substantially increased starting from 35 min as compared to the saline control. The leakage in Bam32$^{-/-}$ mice were minimally increased but significantly less than that in WKYMVm-treated WT mice starting from 40 min. Because of the well-established function of WKYMVm in inducing neutrophil adhesion and emigration as an end-target chemoattractant [36], we simultaneously quantified the numbers of adherent and emigrated neutrophils in the same segment of venule where we determined the permeability indices in both mouse strains before and after the 60-min WKYMVm treatment. Figure 5-2B and 5-2C show that Bam32$^{-/-}$ mice had a significantly lower number of emigrated neutrophils after 60 min treatment with WKYMVm, although the numbers of adherent neutrophils within these groups did not achieve statistical significance. These results reveal that Bam32 plays an important role in WKYMVm-induced microvascular hyperpermeability and neutrophil emigration.

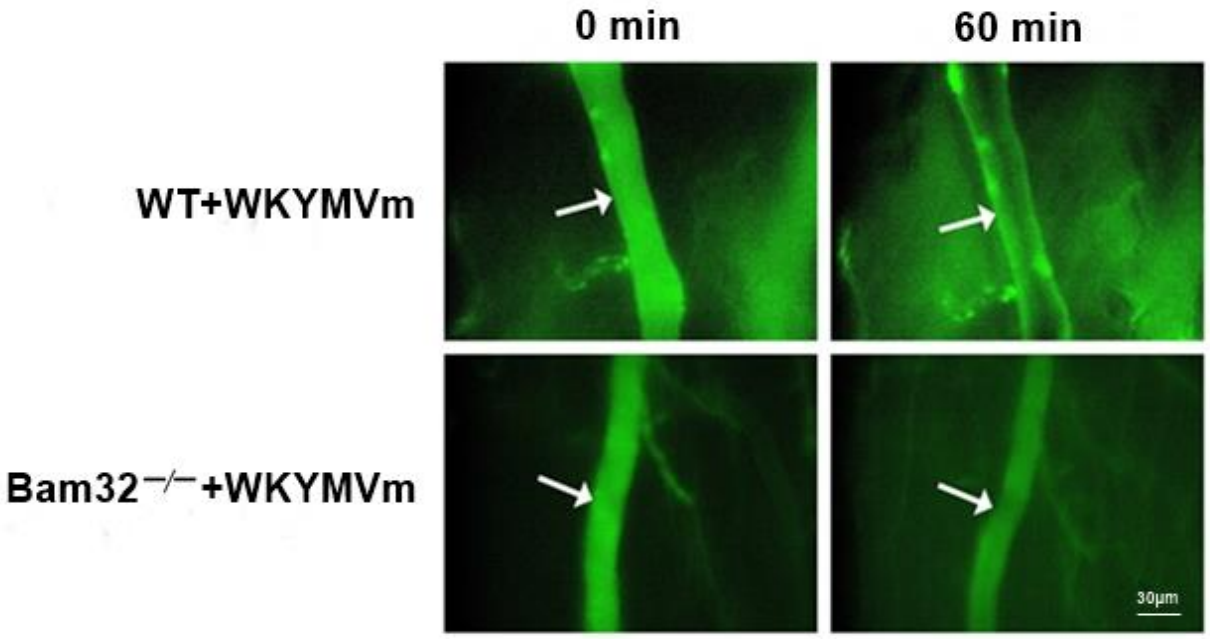

Figure 5-1. WKYMVm-induced microvascular hyperpermeability in WT and Bam32$^{-/-}$ mice (micrographs). Representative fluorescence micrographs of hyperpermeability in mouse cremasteric post-capillary venules before (0 min) and 60 min after superfusion with WKYMVm in WT (upper panel) and Bam32$^{-/-}$ (lower panel) mice. Arrow in micrograph indicates the venular segment for permeability index measurements (Magnification: 200×).

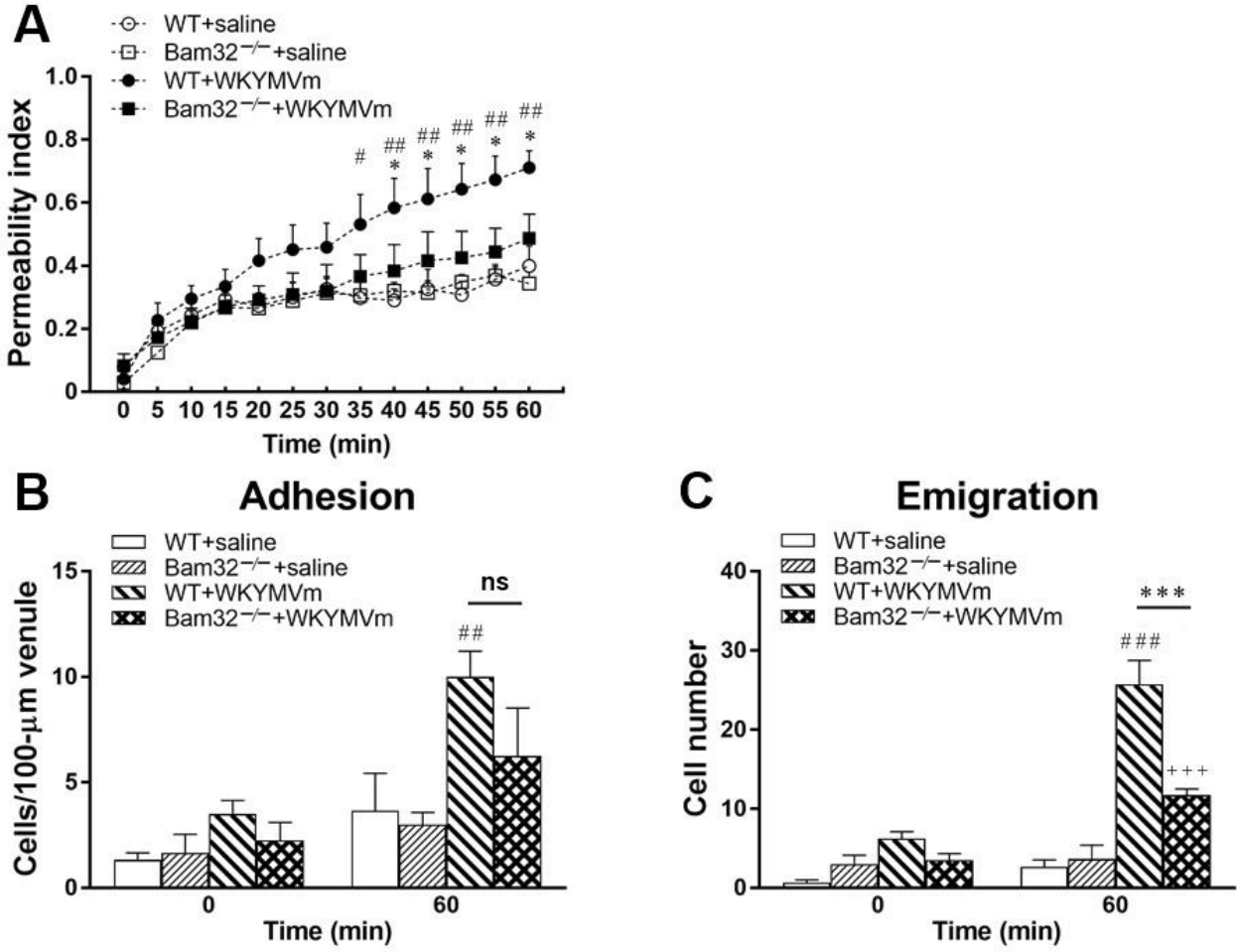

*Figure 5-2. WKYMVm-induced microvascular hyperpermeability in WT and Bam32$^{-/-}$ mice (quantification results). (A) Permeability indices of mouse cremasteric post-capillary venules following 60-min superfusion with bicarbonate-buffered saline (white symbols) or WKYMVm (0.1 μM, black symbols) in WT and Bam32$^{-/-}$ mice. Neutrophil adhesion number on 100-μm length of venule (B) and neutrophil emigration number (C, cells/443×286 μm^2 field) were determined simultaneously in the same experiments of WKYMVm-induced cremasteric microvascular hyperpermeability before (0 min) and 60 min following superfusion with WKYMVm. A–C, mean ± SEM of 4 mice per group. */**/*** indicate significant differences between WT and Bam32$^{-/-}$ mice (*: p<0.05, **: p<0.01 and ***: p<0.001). #/##/### indicate significant differences between WT mice with and without WKYMVm treatment (#: p<0.05, ##: p<0.01 and ###: p<0.001). +++ indicates significant difference between Bam32$^{-/-}$ mice with and without WKYMVm treatment (+++: p<0.001). ns, not significant.*

5.5.2 Deficiency of Bam32 impairs WKYMVm-induced neutrophil emigration in mouse cremaster muscle

Here, we verified the critical role of Bam32 in WKYMVm-induced neutrophil emigration by applying WKYMVm-containing cubes of agarose gel to mouse cremaster muscle, allowing the formation of WKYMVm gradients and scrutinizing the neutrophil recruitment and chemotaxis in both mouse strains. Figure 5-3 shows that deficiency of Bam32 significantly reduced the number of emigrated neutrophils at 30 min and 60 min after the placement of WKYMVm-containing gel, without influencing the number of adherent neutrophils, leukocyte rolling velocity and the number of leukocyte rolling flux. As shown in Figure 5-4, deficiency of Bam32 impaired neutrophil migration distance and neutrophil chemotaxis distance. However, migration velocity, chemotaxis velocity, and chemotaxis index of emigrated neutrophils were not affected. Taken together, these results confirm that WKYMVm-induced neutrophil transmigration is Bam32-dependent. In addition, the decreased migration distance and chemotaxis distance but unaffected velocity indicates that Bam32$^{-/-}$ neutrophils might respond slower to WKYMVm in interstitial chemotaxis as compared to WT neutrophils.

5.5.3 Deficiency of Bam32 transiently increases CXCL2-induced microvascular hyperpermeability

We also substituted the end-target chemoattractant WKYMVm for intermediary chemoattractant CXCL2. In Figure 5-5A, the microvascular permeability in WT mice treated with CXCL2 was significantly elevated at 35 min and onwards when compared to the baseline. Interestingly, the permeability in Bam32$^{-/-}$ mice was elevated dramatically from the baseline as early as 5 min after superfusion with CXCL2 and was even significantly higher than that in CXCL2-treated WT mice during the time period from 15 min to 25 min, although the differences between the two mouse strains disappeared thereafter. Moreover, Figure 5-5B shows that Bam32-dependent, CXCL2-induced differences were diminished by the depletion of mouse circulatory neutrophils. Similarly, we simultaneously measured the numbers of adherent and emigrated neutrophils in the same post-capillary venules where we determined the permeability indices before and after 60-min stimulation with CXCL2. As shown in Figure 5-5C and 5-5D, Bam32$^{-/-}$ mice had no difference in the number of emigrated neutrophils compared to WT mice but had significantly lower number of adherent neutrophils after 60 min stimulation with CXCL2. These distinct responding patterns triggered by CXCL2, together with our previous results of WKYMVm, indicate that Bam32 may through different mechanisms mediate mouse microvascular leakage induced by end-target chemoattractants and intermediary chemoattractants, respectively.

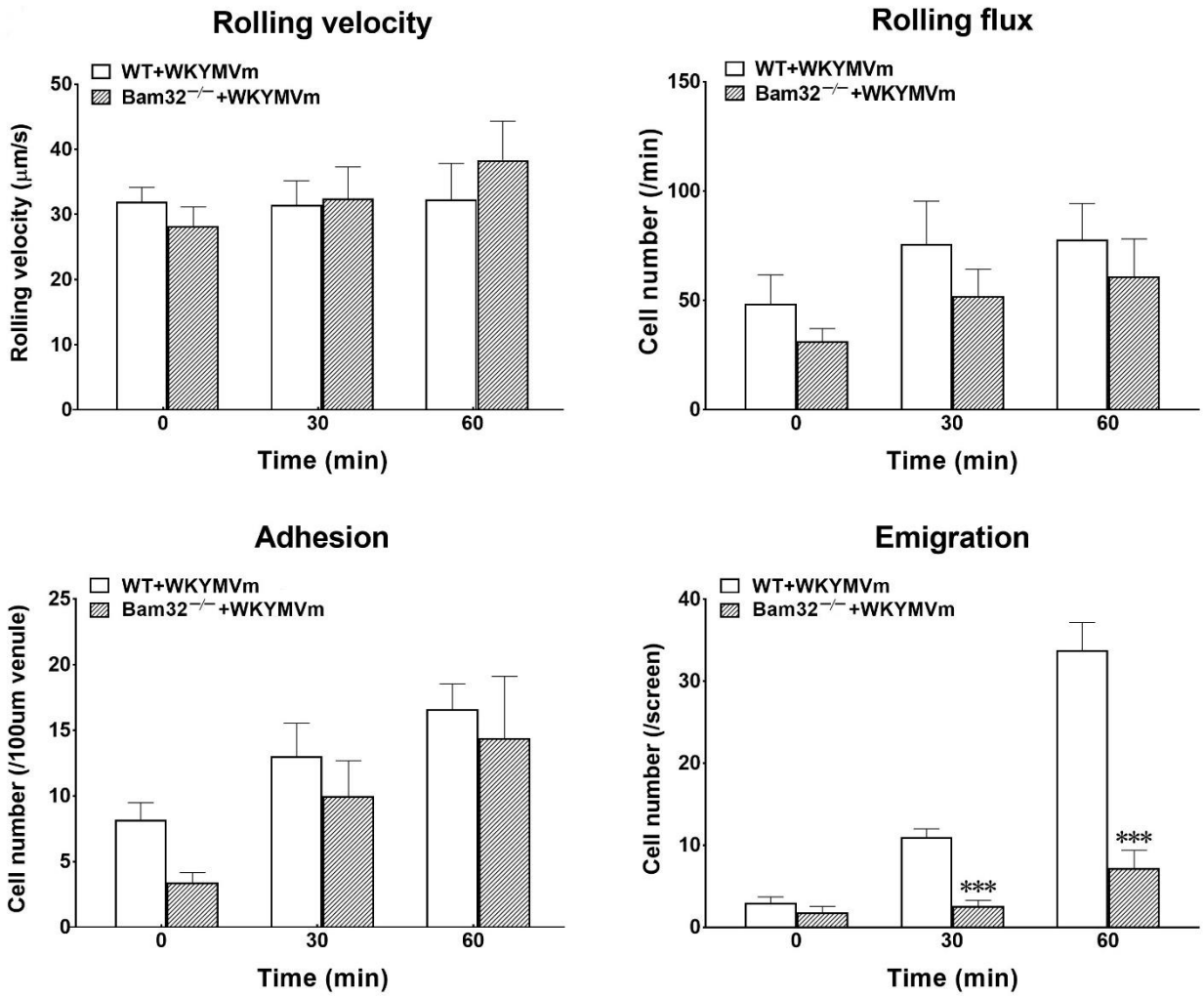

*Figure 5-3. Deficiency of Bam32 decreases WKYMVm-induced neutrophil emigration in mouse cremaster muscle. Leukocyte rolling velocity, leukocyte rolling flux, neutrophil adhesion (cells/100-μm venule), and neutrophil emigration (cells/235 × 208 μm^2 fields) at 30 min and 60 min following the placement and held of WKYMVm-containing gel on cremaster muscle of WT and Bam32$^{-/-}$ mice. Mean ± SEM of 5 mice per group. *** indicates significant differences ($p<0.001$) from WT mice.*

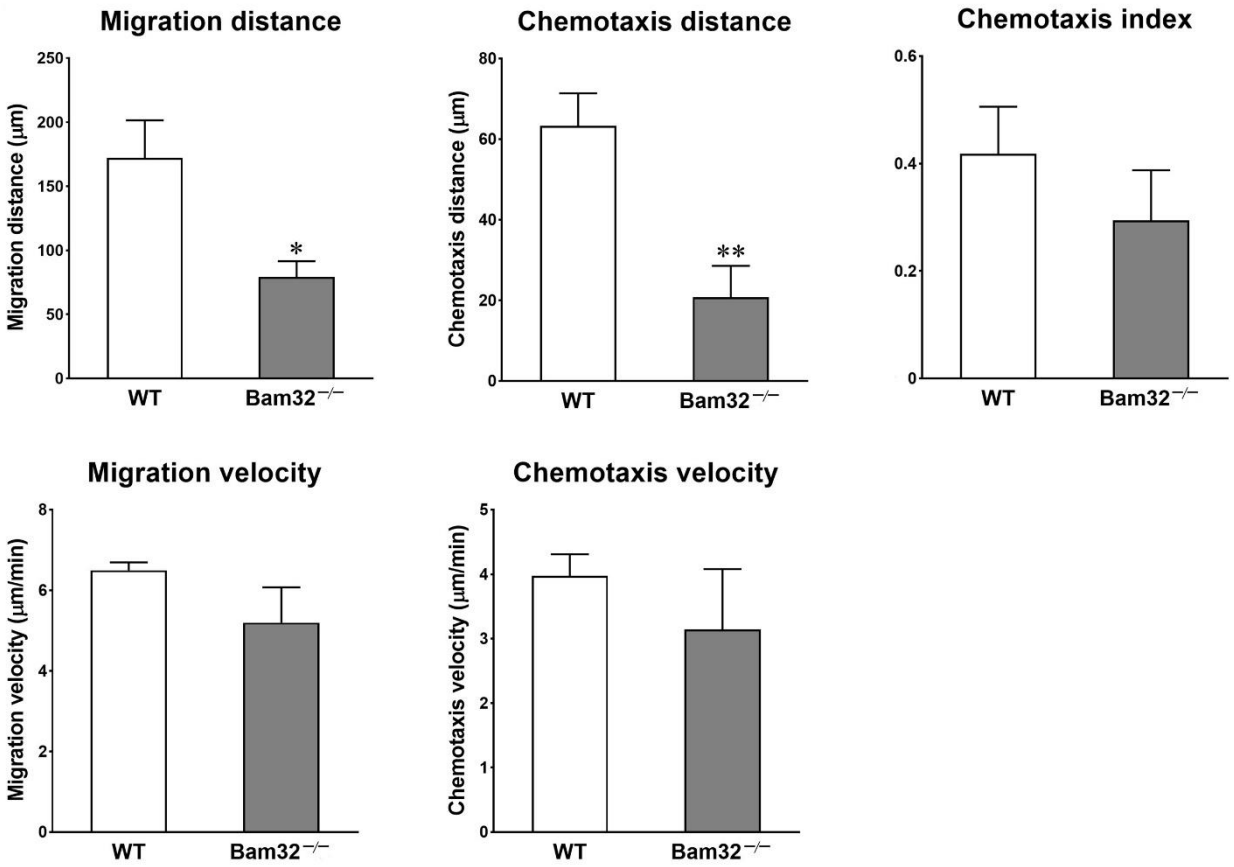

*Figure 5-4. Deficiency of Bam32 decreases WKYMVm-induced neutrophil chemotaxis in mouse cremaster muscle. The migration distance (μm), chemotaxis distance (μm), migration velocity (μm/min), chemotaxis velocity (μm/min) and chemotaxis index of neutrophil chemotaxis in extravascular tissue during 60 min following the placement and held of CXCL2-containing gel on cremaster muscle of WT mice and Bam32$^{-/-}$ mice (averaged from >50 cells). Mean ± SEM of 4 mice per group. * and ** indicate significant differences ($p<0.05$ and $p<0.01$, respectively) from WT mice.*

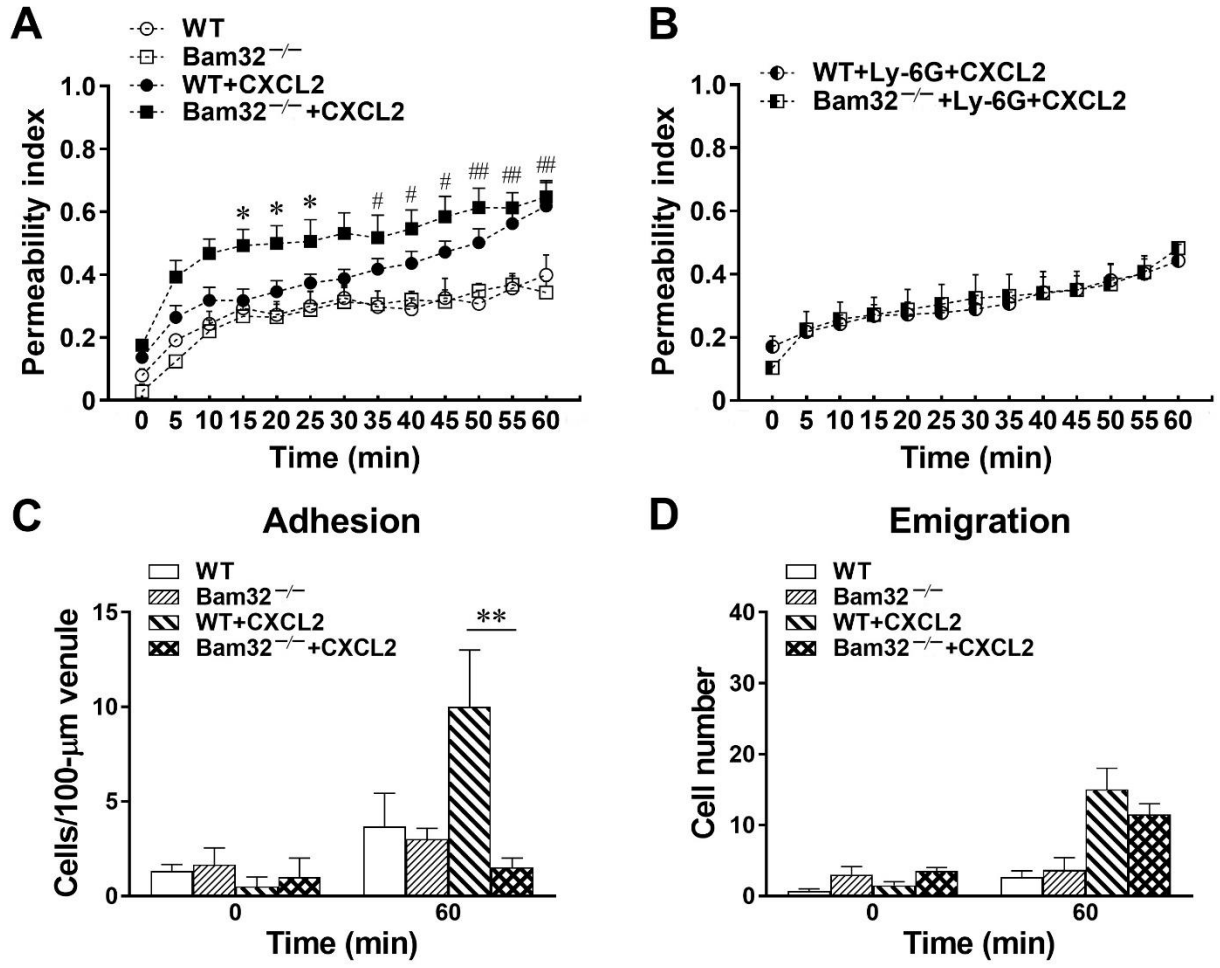

*Figure 5-5. CXCL2-induced microvascular hyperpermeability in WT and Bam32$^{-/-}$ mice. (A) Permeability indices of mouse cremasteric post-capillary venules following 60-min superfusion with bicarbonate-buffered saline (white symbols) or CXCL2 (0.5 nM, black symbols) in WT and Bam32$^{-/-}$ mice. (B) Permeability index of mouse cremasteric post-capillary venules following 60-min superfusion with CXCL2 in the mice with Ly-6G antibody (200 μg, i.p.) 24-h treatment prior to CXCL2 superfusion during experiment of intravital microscopy. Neutrophil adhesion number on 100-μm length of venule (C) and neutrophil emigration number (D, cells/443×286 μm^2 field) determined simultaneously in the same experiments of CXCL2-induced cremasteric microvascular hyperpermeability before (0 min) and 60 min following superfusion with CXCL2. A–D, mean ± SEM of 4 mice per group. */** indicate significant differences between WT and Bam32$^{-/-}$ mice (*: p<0.05 and **: p<0.01). #/## indicate significant differences between WT mice with and without CXCL2 treatment (#: p<0.05 and ##: p<0.01).*

5.5.4 Bam32 deficiency in hematopoietic cells is sufficient to impair WKYMVm-induced microvascular hyperpermeability

Both neutrophils and endothelial cells contribute to the increase of chemoattractant-induced microvascular hyperpermeability. Therefore, the role of Bam32 in specific cell type(s) dominating the changes in permeability needs to be addressed. To distinguish whether the role of Bam32 in WKYMVm-induced microvascular leakage was attributable to endothelial cells or neutrophils, we generated four types of bone marrow chimeric mice (WT→WT, WT→Bam32$^{-/-}$, Bam32$^{-/-}$→WT, and Bam32$^{-/-}$→Bam32$^{-/-}$). We assessed the microvascular leakage in these chimeric mice in response to WKYMVm and found no significant difference in microvascular permeability among four types of chimeric mice without stimulation with WKYMVm (Figure 5-6A). The microvascular permeability in WT→Bam32$^{-/-}$ mice showed a trend of increase compared to WT→WT mice, which shared the same WT neutrophils but had different genotypes of endothelial background, and this reached significance at 15 min after superfusion with WKYMVm (Figure 5-6B). This difference appeared to diminish by 20 min, indicating Bam32 in endothelial cells might play a mild and transient role in elevating WKYMVm-induced microvascular leakage at early time points. Interestingly, after 30-min superfusion with WKYMVm, the microvascular permeability in WT→WT mice was significantly higher than that in Bam32$^{-/-}$→WT mice which had the same WT endothelial background without Bam32 in neutrophils. Similarly, the microvascular permeability in WT→Bam32$^{-/-}$ mice was also significantly higher than that in Bam32$^{-/-}$→ Bam32$^{-/-}$ mice, demonstrating that Bam32 in neutrophils dominates in regulating WKYMVm-induced microvascular hyperpermeability regardless of the endothelium with or without Bam32. Figure 5-6C and 5-6D show that the number of adherent neutrophils in Bam32$^{-/-}$→Bam32$^{-/-}$ mice was significantly lower than that in WT→Bam32$^{-/-}$ mice after 60-min superfusion with WKYMVm, revealing a possible role of neutrophil adhesion in regulating microvascular leakage. Moreover, the number of emigrated neutrophils in Bam32$^{-/-}$→Bam32$^{-/-}$ mice was significantly lower than that in chimeric mouse strains with WT neutrophils (WT→WT, WT→ Bam32$^{-/-}$) after 60-min superfusion with WKYMVm, indicating that Bam32 in neutrophils is critically important in regulating WKYMVm-induced neutrophil emigration, whereas Bam32 in endothelial cells may play a secondary role in this process. These results from chimeric mice strongly suggest that Bam32 in recruited neutrophils rather than in endothelial cells is required in WKYMVm-induced microvascular hyperpermeability.

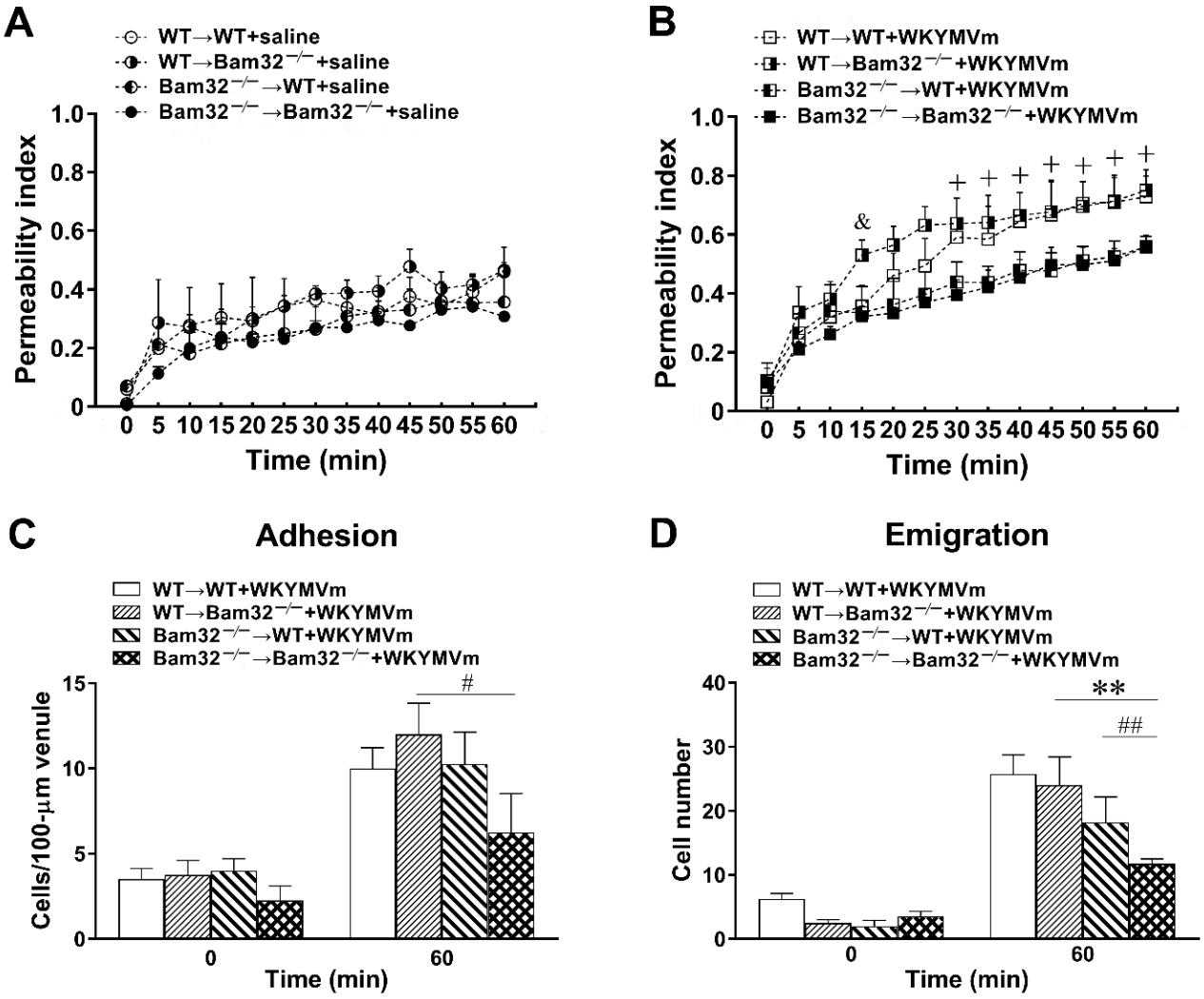

*Figure 5-6. WKYMVM-induced microvascular hyperpermeability in chimeric mice. Permeability indices of mouse cremasteric post-capillary venules following 60-min superfusion with bicarbonate-buffered saline (A) or WKYMVm (B, 0.1 μM) in chimeric mice. Neutrophil adhesion number on 100-μm length of venule (C) and neutrophil emigration number (D, cells/443×286 μm² field) were determined simultaneously in the same experiments of WKYMVm-induced cremasteric microvascular hyperpermeability before (0 min) and 60 min following superfusion with WKYMVm. A–D, mean ± SEM of 3 mice per group. & indicates a significant difference between WT→WT and WT→Bam32$^{-/-}$ mice (&: p<0.05). + indicates significant differences between WT→WT and Bam32$^{-/-}$→WT mice (+: p<0.05). ** indicates a significant difference between WT→WT and Bam32$^{-/-}$→Bam32$^{-/-}$ mice (**: p<0.01). #/## indicate significant differences between WT→Bam32$^{-/-}$ and Bam32$^{-/-}$→Bam32$^{-/-}$ mice (#: p<0.05 and ##: p<0.01).*

5.5.5 Bam32 is required for WKYMVm-induced intracellular and extracellular ROS production in neutrophils

Acute elevation in mouse microvascular permeability is attributed to many neutrophil-related factors, such as the production of ROS. A series of experiments were performed to determine the intracellular and extracellular production of ROS in bone marrow-derived neutrophils from WT and Bam32$^{-/-}$ mouse strains *in vitro*. Figure 5-7A shows that deficiency of Bam32 significantly reduced the production of intracellular ROS in mouse neutrophils starting from 10-min stimulation with WKYMVm. Moreover, stimulation with PMA substantially increased the production of ROS in neutrophils but the differences between two mouse strains became significant only after 40-min stimulation with WKYMVm. This indicates that Bam32 may play a more important role in WKYMVm-induced rather than PMA-induced ROS generation in neutrophils. Moreover, extracellular ROS production in Bam32$^{-/-}$ neutrophils was significantly lower than that in WT neutrophils at 10 min and 20 min following stimulation with WKYMVm (Figure 5-7B), further supporting the critical role of Bam32 in neutrophil ROS generation triggered by WKYMVm. As shown in Figure 5-7C, Bam32 deficiency also impaired the basal and WKYMVm-induced activation of Rac1. Together, these data suggest an unreported role for Bam32 in ROS generation in neutrophils.

5.5.6 Bam32 is not required for CXCL2-induced ROS production in neutrophils

As Bam32-dependent ROS production is critically important in WKYMVm-induced microvascular hyperpermeability, whether CXCL2-induced ROS generation is Bam32 dependent needs to be determined as a matter of course. In this study, we measured CXCL2-induced intracellular ROS generation in isolated bone marrow-derived WT and Bam32$^{-/-}$ neutrophils. Where indicated, TNFα (1 nM) was applied 20 min prior to the stimulation of CXCL2 for priming neutrophils as described in previous studies [251,385–388]. As depicted in Figure 5-8, stimulation with CXCL2 significantly increased ROS production in TNFα-primed WT neutrophils at 20 min and 40 min, whereas in TNFα-primed Bam32$^{-/-}$ neutrophils upon the stimulation and at 10 min, indicating that CXCL2 can trigger ROS production in TNFα-primed neutrophils. Moreover, priming with TNFα significantly increased ROS production in CXCL2-treated WT neutrophils at 20 min, 30 min, and 40 min, whereas in CXCL2-treated Bam32$^{-/-}$ neutrophils after 10 min and on, in line with the well-established functions of TNFα priming. Surprisingly, deficiency of Bam32 does not result in significant difference in CXCL2-induced intracellular ROS production in neutrophils with or without TNFα priming, indicating that CXCL2-induced ROS production is Bam32 independent.

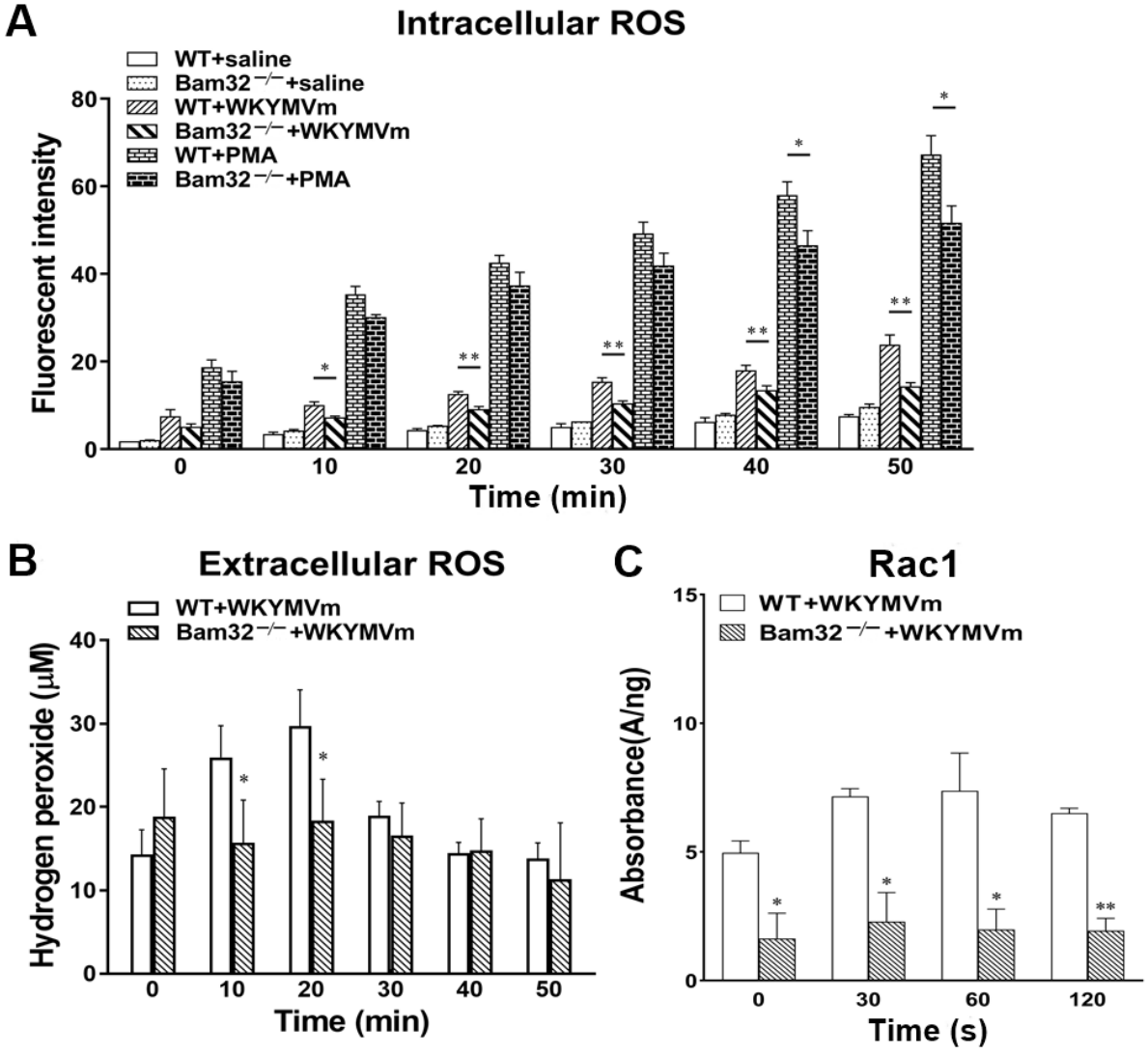

*Figure 5-7. ROS generation and Rac1 activation in bone marrow-derived neutrophils from WT and Bam32$^{-/-}$ mice. (A) Time course of intracellular generation of ROS in isolated neutrophils with saline, WKYMVm (0.1 μM) or PMA (0.2 μM). (B) Time course of extracellular generation of ROS in isolated neutrophils with WKYMVm. (C) Rac1 activation in isolated neutrophils with WKYMVm. A–C, mean ± SEM of 3–4 samples per group. Each sample was collected from one mouse (A and B) or two mice (C) of the same strain. The measurements were duplicated in the 96-well plate. */** indicate significant differences between WT and Bam32$^{-/-}$ neutrophils (*: $p<0.05$ and **: $p<0.01$). Fluorescent signals at 0 min in panel A and B were measured immediately following the addition of saline, WKYMVm or PMA. Fluorescent signals at 0 min in panel C were measured in neutrophils without the treatment of WKYMVm.*

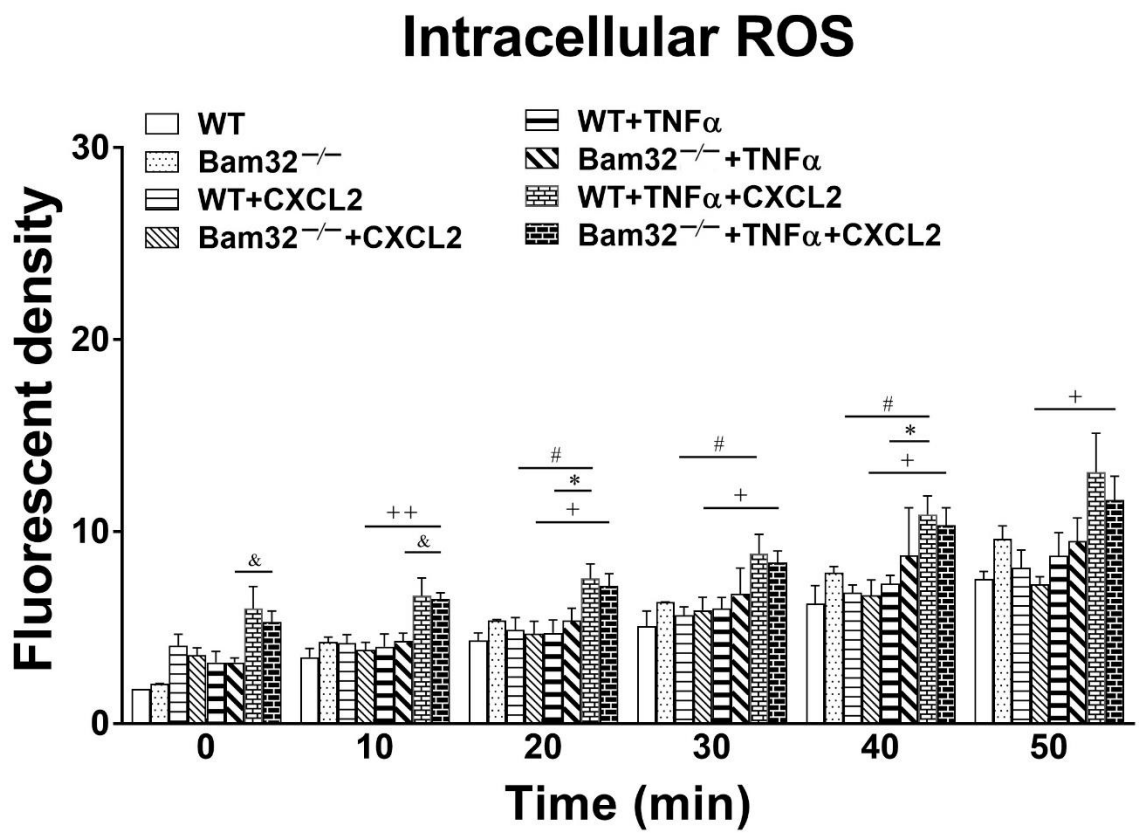

*Figure 5-8. Time course of intracellular CXCL2 (0.5 nM)-induced generation of ROS in isolated neutrophils primed with or without TNFα (12 pM). Mean ± SEM of 4 mice per group. The measurements were duplicated in the 96-well plate. * indicates significant differences between WT groups with or without CXCL2 treatment. (*: $p<0.05$). # indicates significant differences between WT groups with or without TNFα priming. (#: $p<0.05$). & indicates significant differences between Bam32$^{-/-}$ groups with or without CXCL2 treatment. (&: $p<0.05$). +/++ indicate significant differences between Bam32$^{-/-}$ groups with or without TNFα priming. (+: $p<0.05$ and ++: $p<0.01$). Fluorescent signals at 0 min were measured immediately following the addition of saline or CXCL2.*

5.5.7 Bam32-dependent production of ROS promotes WKYMVm-induced microvascular hyperpermeability

To verify the importance of Bam32-dependent ROS production in WKYMVm-induced mouse microvascular leakage, we applied an NADPH oxidase inhibitor DPI or a hydrogen peroxide metabolizing enzyme catalase to WKYMVm-superfused mouse cremaster muscle. As depicted in Figure 5-9A, WKYMVm-induced microvascular hyperpermeability in WT mice was significantly higher than that in Bam32$^{-/-}$ mice, however, the application of DPI alleviated the leakage in WT mice and completely eliminated the differences in permeability changes between two mouse strains. Similarly, the application of catalase not only substantially reduced the microvascular leakage in WT mice, but also eliminated the differences in permeability changes between two mouse strains (Figure 5-10A). In contrast, DPI treatment did not impact the numbers of adherent and emigrated neutrophils in WT or Bam32$^{-/-}$ mice, whereas deficiency of Bam32 significantly decreased the numbers of adherent and emigrated neutrophils after 60-min of WKYMVm treatment when DPI was present (Figure 5-9B and 5-9C). Moreover, the application of catalase did not significantly change the numbers of adherent and emigrated neutrophils induced by WKYMVm in either mouse strains, except for increased number of adherent neutrophils in WKYMVm-treated Bam32$^{-/-}$ mice for unknown reason (Figure 5-10B and 5-10C). These results reveal that Bam32-dependent ROS production in recruiting neutrophils, functionally independent of neutrophil adhesion and emigration, is critically required in WKYMVm-induced microvascular hyperpermeability.

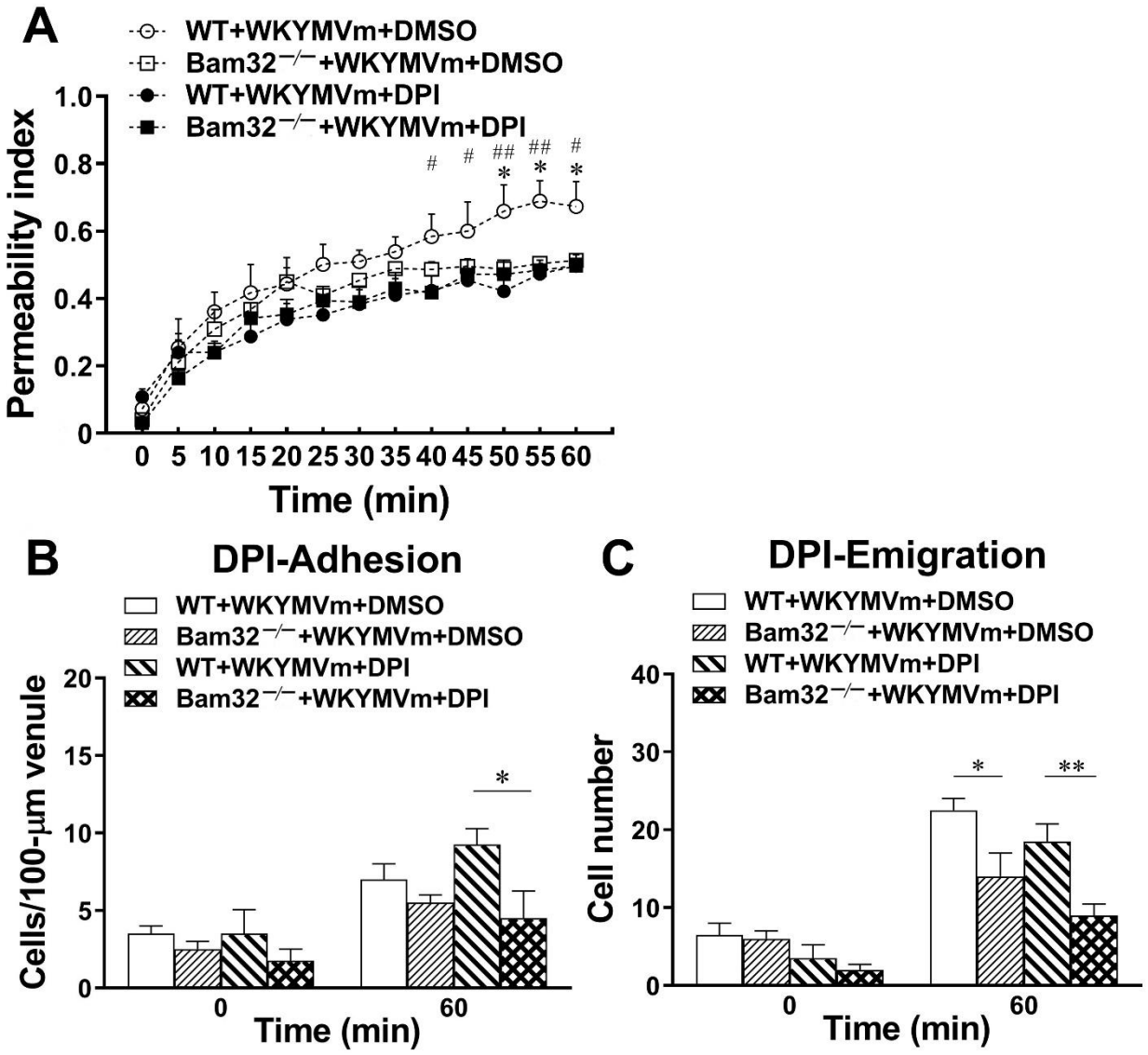

*Figure 5-9. Effect of DPI on WKYMVm-induced microvascular hyperpermeability. (A) Permeability indices of mouse cremasteric post-capillary venules following 60-min WKYMVm (0.1 μM) superfusion in the presence of DMSO (vehicle) or DPI (40 μM) for 30 min prior to and during 60-min WKYMVm superfusion. Neutrophil adhesion number of 100-μm length of venule (B) and neutrophil emigration number (C, cells/443×286 μm² field) were determined simultaneously in the same experiments of WKYMVm-induced cremasteric microvascular hyperpermeability before (0 min) and 60 min following superfusion with WKYMVm in the presence of DMSO or DPI. A–C, mean ± SEM of 4 mice per group. */** indicate significant differences between WT and Bam32$^{-/-}$ mice (*: p<0.05 and **: p<0.01). #/## indicate significant differences between WT mice with or without DPI or functional catalase (#: p<0.05 and ##: p<0.01).*

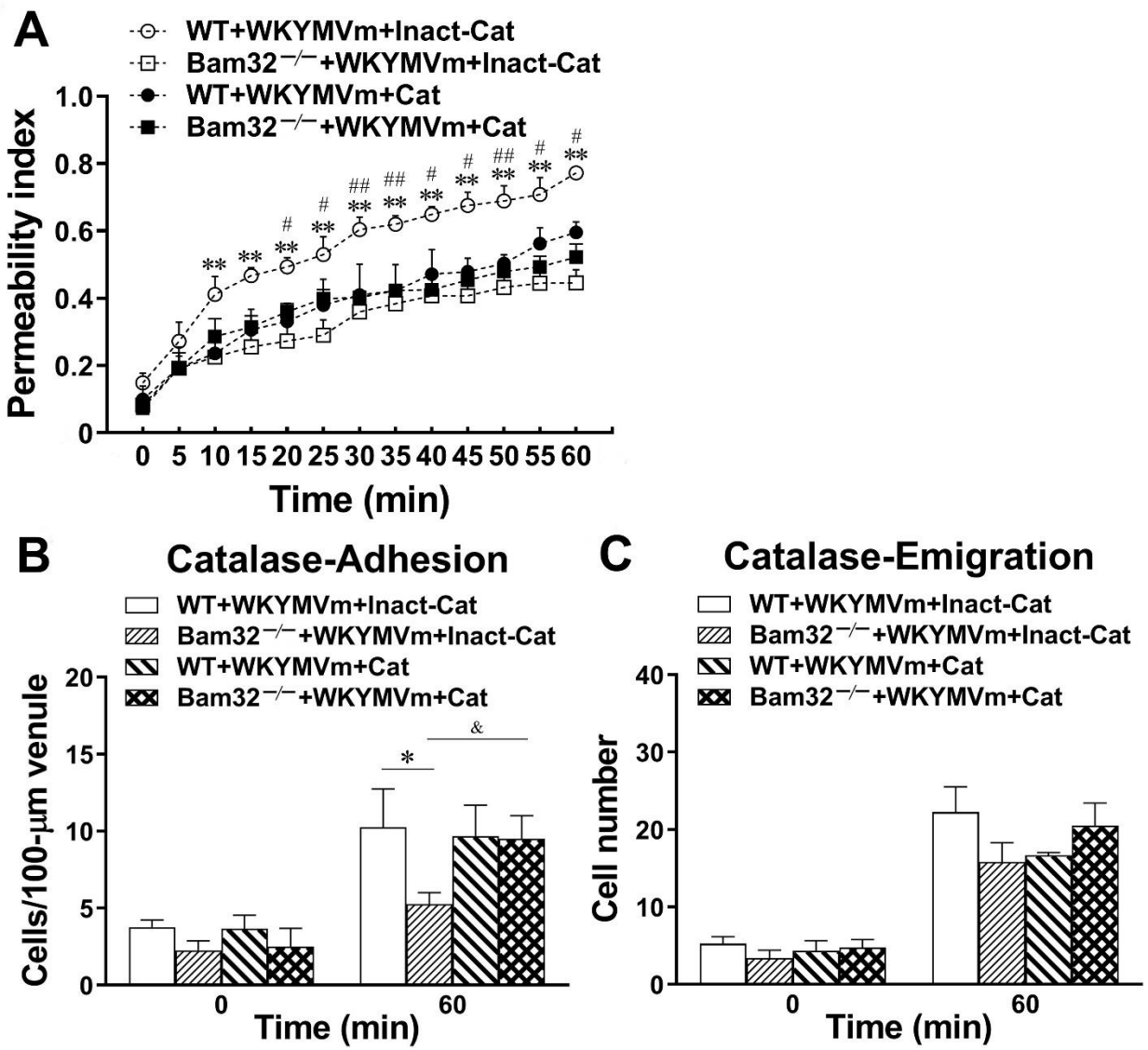

*Figure 5-10. Effect of catalase on WKYMVm-induced microvascular hyperpermeability. (A) Permeability indices of mouse cremasteric post-capillary venules following 60-min WKYMVm superfusion in the presence of inactivated catalase (Inact-Cat) or functional catalase (Cat, 200 units/mL) for 30 min prior to and during 60-min WKYMVm superfusion. Neutrophil adhesion number of 100-μm venule (B) and neutrophil emigration number (C, cells/443×286 μm² field) were determined simultaneously in the same experiments of WKYMVm-induced cremasteric microvascular hyperpermeability before (0 min) and 60 min following superfusion with WKYMVm in the presence of inactivated catalase or functional catalase. A–C, mean ± SEM of 6 mice per group. */** indicate significant differences between WT and Bam32$^{-/-}$ mice (*: $p<0.05$ and **: $p<0.01$). #/## indicate significant differences between WT mice with or without DPI or functional catalase (#: $p<0.05$ and ##: $p<0.01$). & indicates a significant difference between Bam32$^{-/-}$mice with functional catalase and with inactivated catalase (&: $p<0.05$).*

5.5.8 Deficiency of Bam32 impairs WKYMVm-induced phosphorylation of ERK1/2 in neutrophils without influencing phosphorylation of p38 and JNK

Given that WKYMVm activates three downstream MAPK cascades, namely p38, ERK1/2 and JNK through FPR [389], we further tested the possible involvement of these MAPK signaling pathways in Bam32$^{-/-}$ neutrophils. As shown in Figure 5-11 and 5-12, deficiency of Bam32 failed to impact the phosphorylation or total levels of p38 or JNK, but significantly impaired the phosphorylation of ERK1/2 after 2-min and 5-min stimulation with WKYMVm. These results indicate that the impairment of ERK1/2 signaling pathway may be involved in Bam32-dependent production of ROS in WKYMVm-stimulated mouse neutrophils.

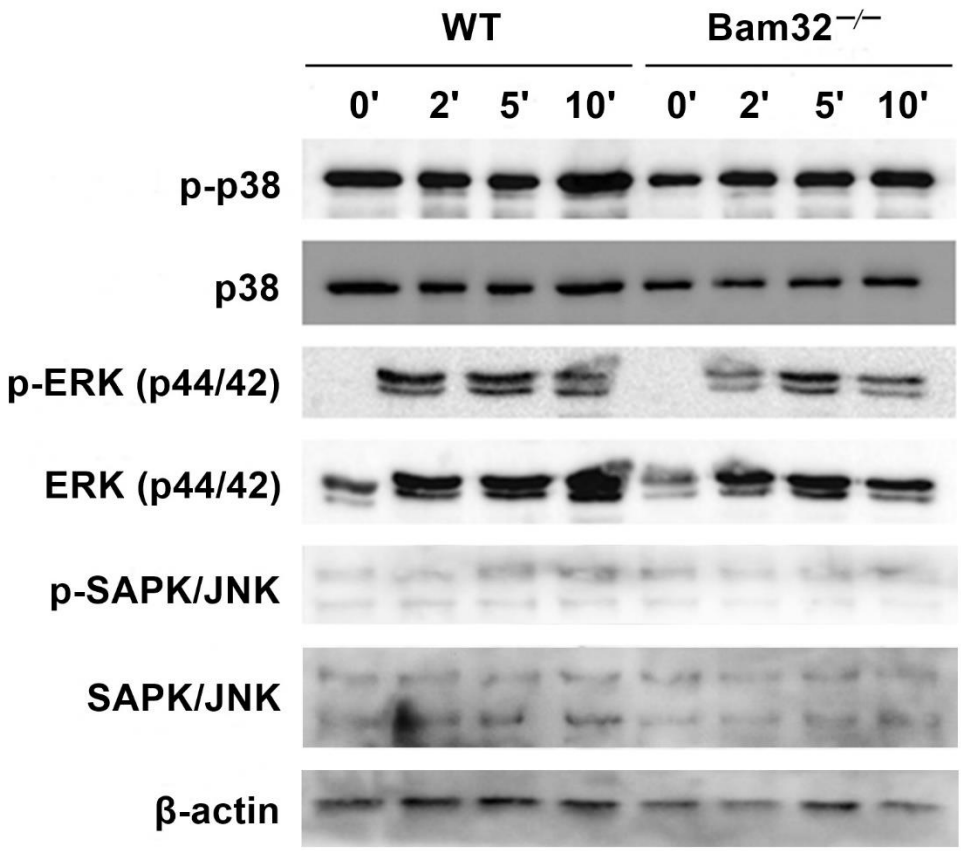

Figure 5-11. WKYMVm-induced phosphorylation of p38, ERK1/2 and JNK MAPK in isolated WT and Bam32$^{-/-}$ neutrophils (blots). Representative original Western blots of phosphorylated and total p38, ERK1/2 and JNK MAPK determined in bone marrow-derived neutrophils from WT and Bam32$^{-/-}$ mice before (0 min) and 2–10 min after treatment with WKYMVm (0.1 μM).

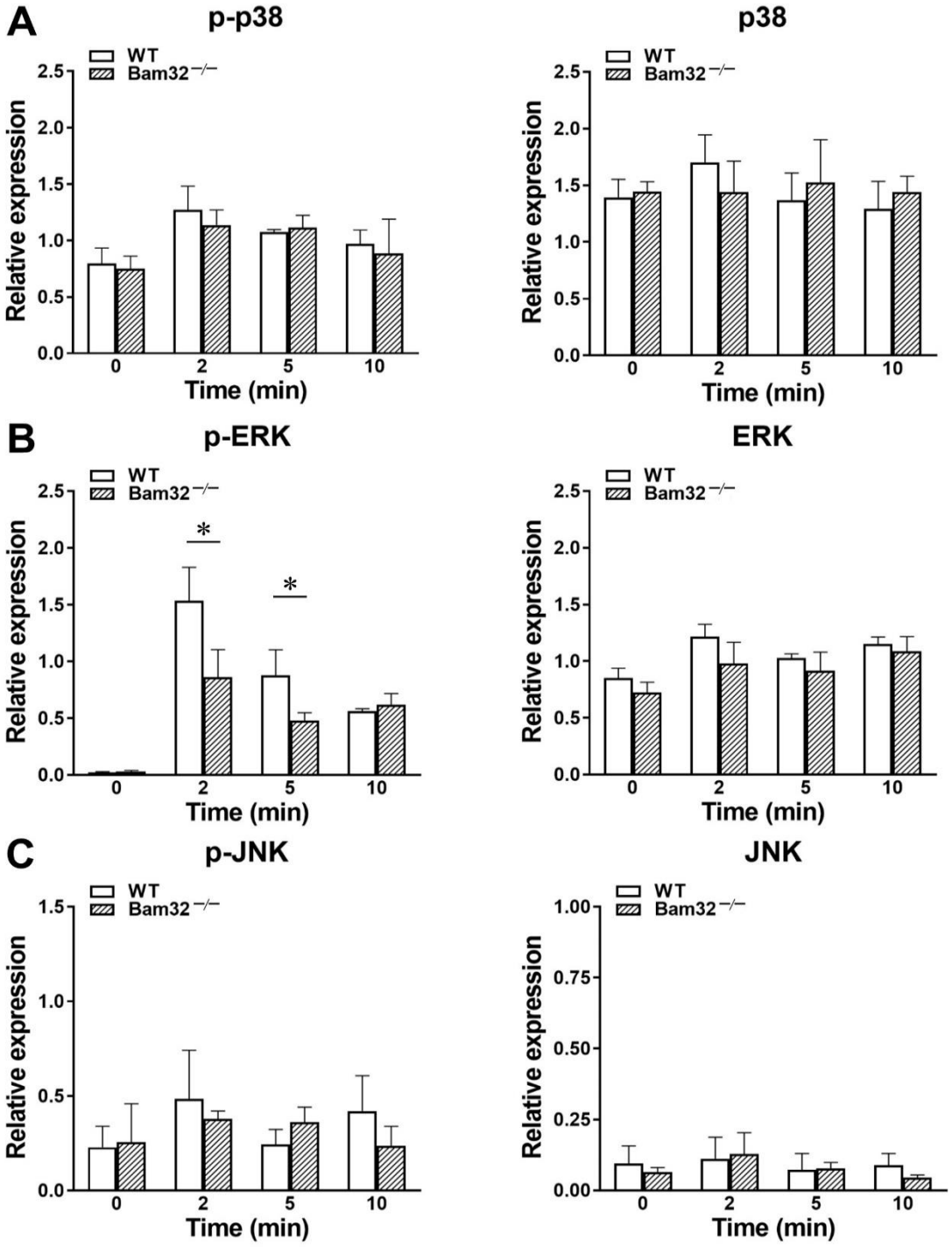

*Figure 5-12. WKYMVm-induced phosphorylation of p38, ERK1/2 and JNK MAPK in isolated WT and Bam32$^{-/-}$ neutrophils (quantification results). Levels of phosphorylated (p) and total p38 (A), ERK1/2 (B) and JNK (C) determined in bone marrow-derived neutrophils from WT and Bam32$^{-/-}$ mice before (0 min) and 2–10 min after treatment with WKYMVm (0.1 μM). Mean ± SEM of 3 samples per group. Each sample was collected from two mice of the same strain. * indicates significant differences between WT and Bam32$^{-/-}$ neutrophils.*

5.5.9 Pharmacological inhibition of ERK1/2 signaling pathway suppresses WKYMVm-induced intracellular ROS production *in vitro* and microvascular hyperpermeability *in vivo*

To verify the possible involvement of ERK1/2 signaling in neutrophil ROS generation and microvascular leakage, we determined the effects of the NADPH oxidase inhibitor DPI, the ERK1/2 signaling inhibitors BVD-523 and PD98059 on WKYMVm-induced intracellular ROS generation, microvascular hyperpermeability, and ERK1/2 phosphorylation. We found that DPI and BVD-523 significantly reduced basal and WKYMVm-induced generation of intracellular ROS in WT neutrophils at all time points tested, whereas PD98059 only suppressed WKYMVm-triggered ROS generation at 10 min (Figure 5-13), indicating the involvement of ERK1/2 activation in neutrophil ROS generation at early time. Moreover, pre-superfusion of mouse cremaster muscle with BVD-523 or PD98059 alleviated WKYMVm-induced microvascular hyperpermeability starting from 40 min and 55 min respectively (Figure 5-14A) and reduced the number of emigrated neutrophils in WKYMVm-treated WT mice without changing the number of adherent neutrophils (Figure 5-14B and 5-14C), supporting that the ERK1/2-involving ROS generation regulates WKYMVm-induced microvascular hyperpermeability. Interestingly, Figure 5-15 shows that inhibition of ROS production by DPI resulted in reduced ERK1/2 phosphorylation in WKYMVm-stimulated WT neutrophils, suggesting that ROS production may also contribute to the phosphorylation of ERK1/2. On the other hand, the application of BVD-523 markedly increased WKYMVm-induced ERK1/2 phosphorylation (Figure 5-15), consistent with previously published data showing that this inhibitor increases ERK1/2 phosphorylation while strongly inhibiting its activity in phosphorylating substrates [381]. In addition, BVD-523 (5 μM) completely inhibit intracellular ROS generation in neutrophils treated with PMA, and to a much lesser extent, with WKYMVm (Figure 5-16). Therefore, BVD-523 at this dose may not be specific enough to WKYMVm-induced, MAPK-mediated ROS generation, but may also inhibit PMA-induced, PKC-mediated ROS generation in neutrophils. In brief, these results strongly suggest that Bam32-dependent ERK1/2 signaling pathway is crucial in WKYMVm-induced neutrophil ROS generation and subsequent neutrophil ROS-mediated increases in microvascular leakage.

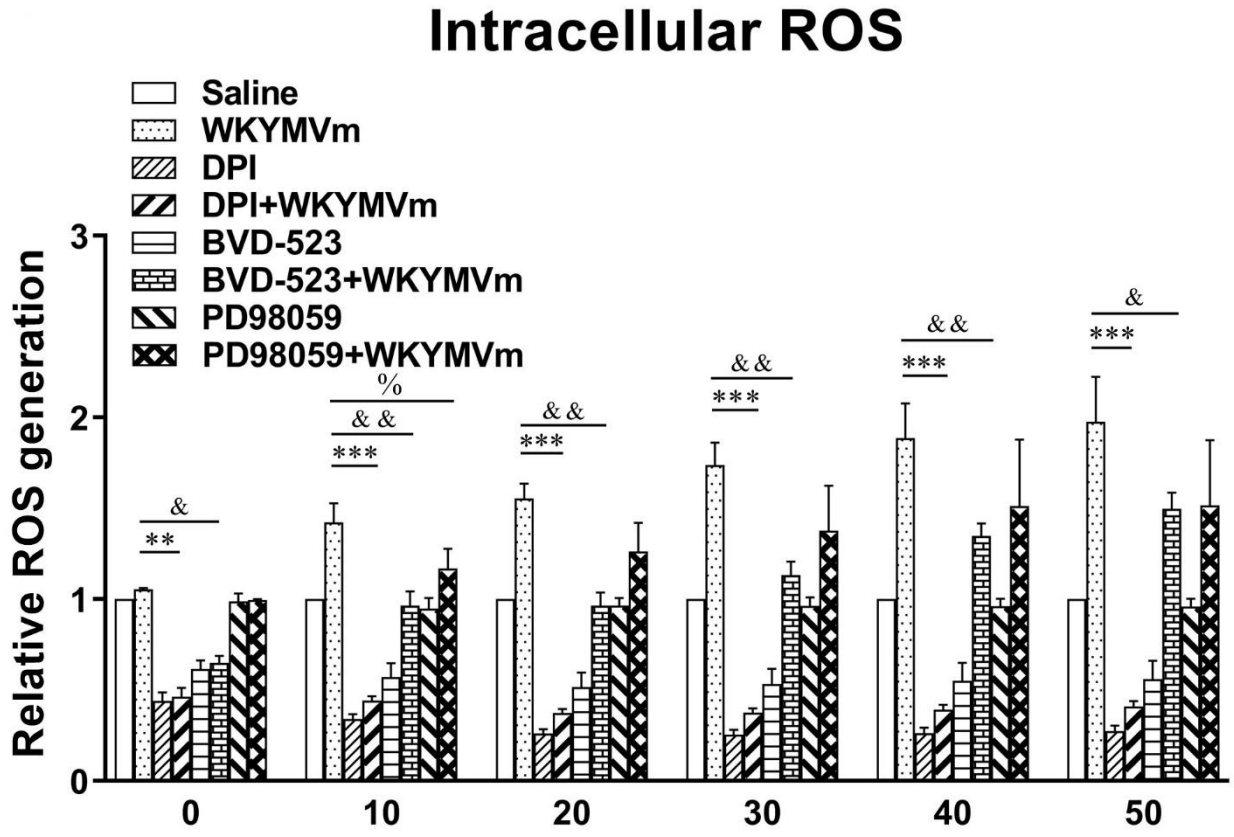

*Figure 5-13. Pharmacological inhibition of ERK1/2 signaling in WKYMVm-induced intracellular ROS generation. Time course of intracellular generation of ROS in isolated neutrophils pre-incubated with vehicle (DMSO), DPI (40 μM), BVD-523 (5 μM) and PD98059 (50 μM), respectively, for 30 min prior to and during 60-min treatment of WT neutrophils with saline or WKYMVm (0.1 μM). Mean ± SEM of 3–4 mice per group. The measurements were duplicated in the 96-well plate. **/*** indicate significant differences between WKYMVm-treated WT neutrophils with and without DPI (**: $p<0.01$ and ***: $p<0.001$). &/&& indicate significant differences between WKYMVm-treated WT neutrophils with and without BVD-523 (&: $p<0.05$ and &&: $p<0.01$). % indicates significant difference between WKYMVm-treated WT neutrophils with and without PD98059 (%: $p<0.05$).*

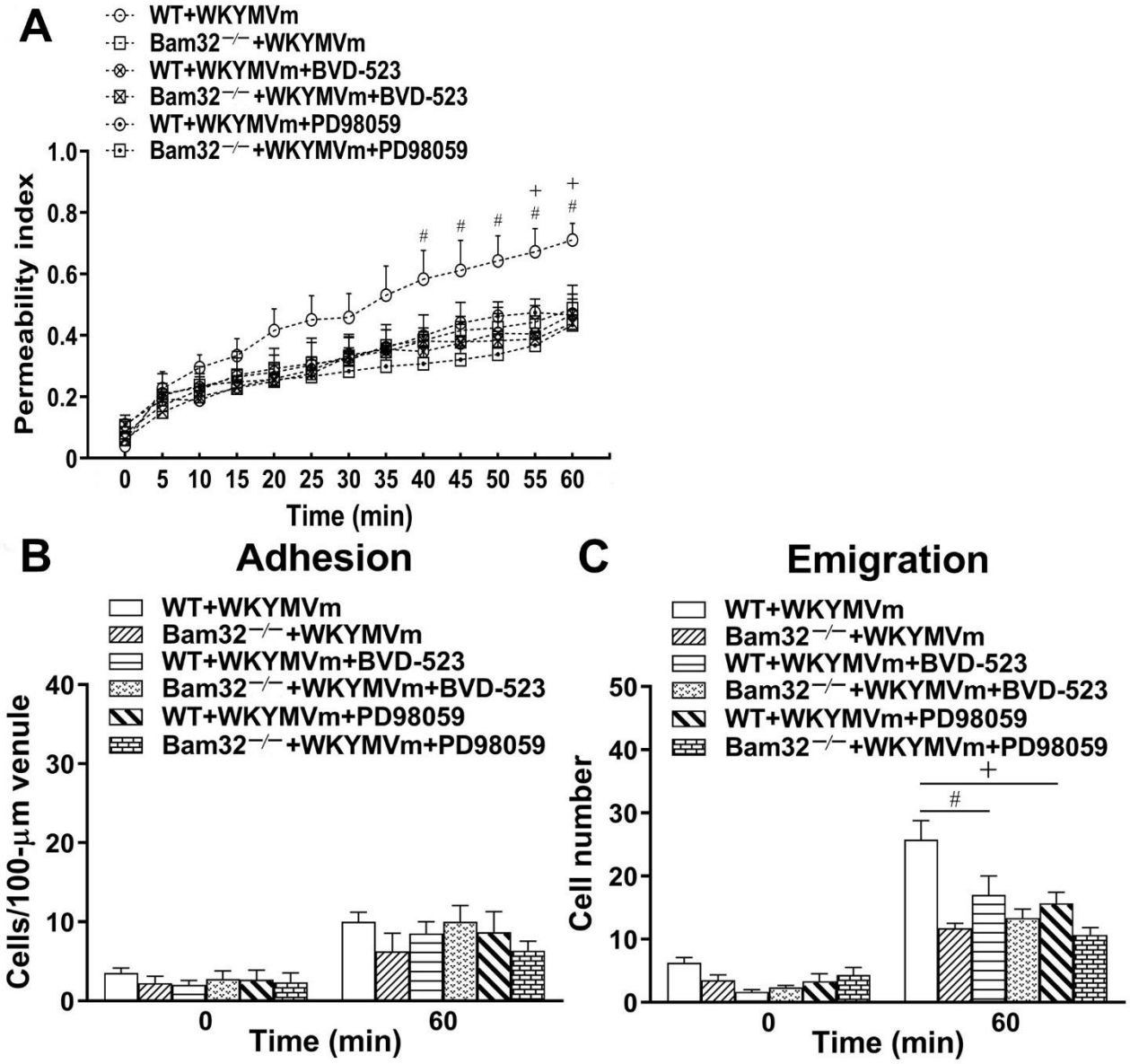

Figure 5-14. Pharmacological inhibition of ERK1/2 signaling in WKYMVm-induced intracellular microvascular hyperpermeability. (A) Permeability indices of mouse cremasteric post-capillary venules following 60-min WKYMVm superfusion in the absence or presence of BVD-523 or PD98059 for 30 min prior to and during 60-min WKYMVm superfusion in WT and Bam32$^{-/-}$ mice. Neutrophil adhesion number on 100-μm length of venule (B) and neutrophil emigration number (cells/443×286 μm^2 field, C) were determined simultaneously in the same experiments of WKYMVm-induced cremasteric microvascular hyperpermeability before (0 min) and 60 min following superfusion with WKYMVm in the absence or presence of BVD-523 or PD98059 in the two mouse strains. A–C, mean ± SEM of 4 mice per group. # indicates significant differences between WKYMVm-treated WT mice with and without BVD-523 (#: $p<0.05$). + indicates significant differences between WKYMVm-treated WT mice with and without PD98059 (+: $p<0.05$).

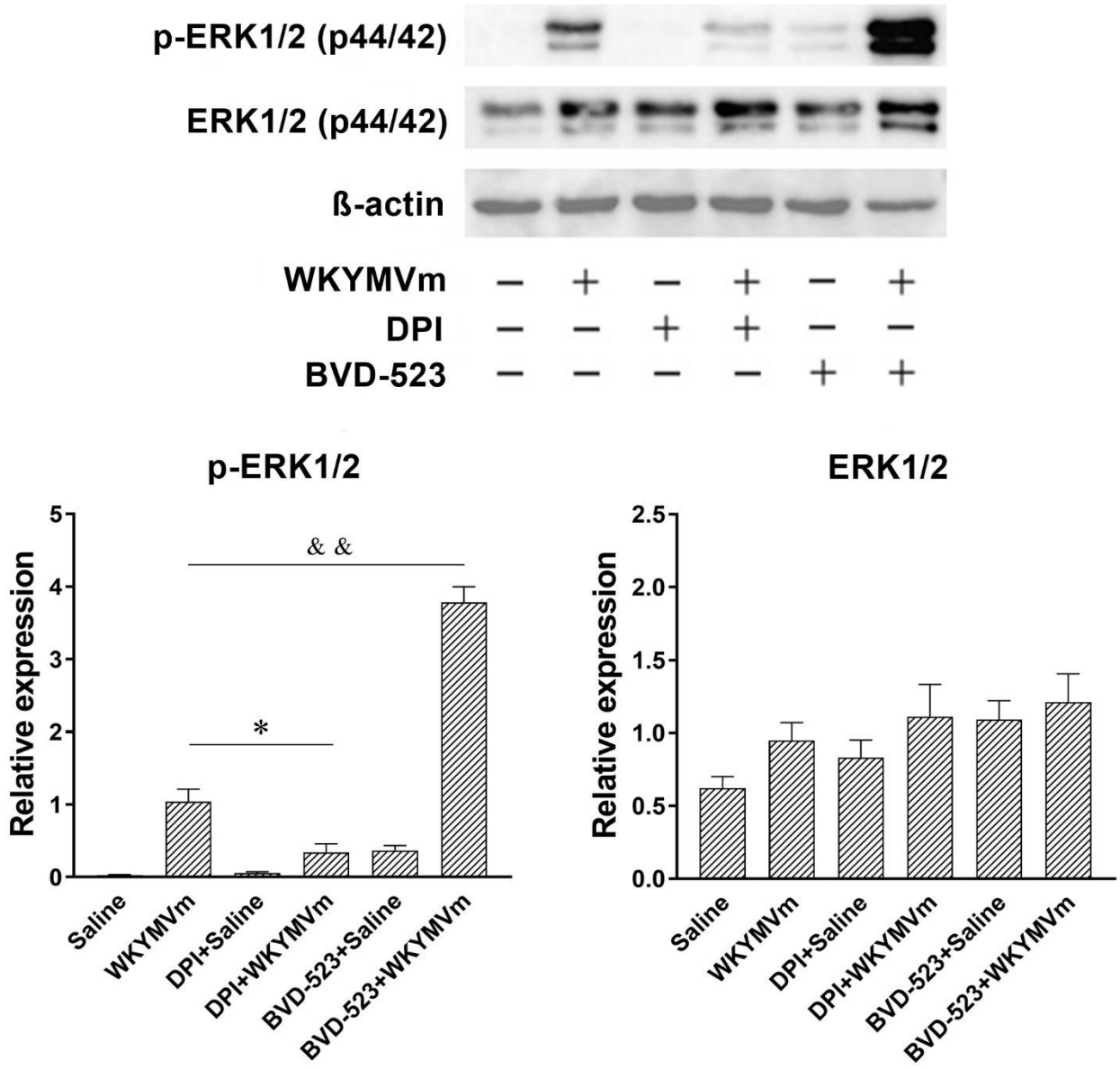

*Figure 5-15. Pharmacological inhibition of ROS generation in WKYMVm-induced phosphorylation of ERK1/2. Original Western blots representative of at least three independent experiments and the quantification data of phosphorylated and total ERK1/2 determined in bone marrow-derived WT neutrophils pre-incubated with vehicle, DPI or BVD-523 for 30 min prior to the 2-min treatment of WT neutrophils with saline or WKYMVm. Mean ± SEM of 3–4 samples per group. Each sample was collected from two mice of the same strain. * indicates significant difference between WKYMVm-treated WT neutrophils with and without DPI (*: $p<0.05$). && indicates significant difference between WKYMVm-treated WT neutrophils with and without BVD-523 (&&: $p<0.01$).*

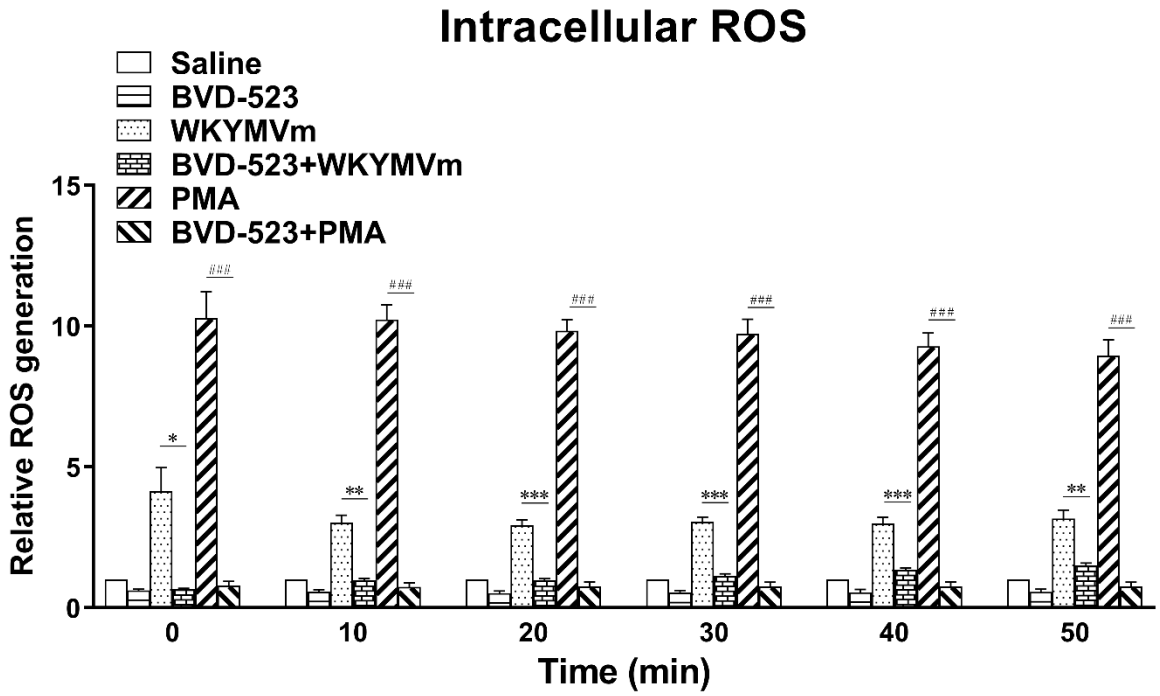

*Figure 5-16. Effect of ERK1/2 inhibitor BVD-523 on intracellular ROS production in WT neutrophils treated with WKYMVm (0.1 μM) or PMA (0.2 μM). Mean ± SEM of 3–4 mice per group. The measurements were duplicated in the 96-well plate. **/*** indicate significant differences between WKYMVm-stimulated groups with and without BVD-523 (**: p<0.01 and ***: p<0.001). ### indicate significant differences between PMA-stimulated groups with and without BVD-523 (###: p<0.001).*

5.6 Discussion

In this study, we unravel a molecular mechanism for Bam32-dependent production of ROS through ERK1/2 signaling by neutrophils with functional impact in WKYMVm-induced microvascular hyperpermeability. Since the endothelium has the capability of inducing local oxidative stress to impair the microvascular barrier, we scrutinized the role of Bam32 in neutrophils by determining the microvascular hyperpermeability using bone marrow chimeric mice. Moreover, we found the evidence that Bam32 plays a role in WKYMVm-induced activation of ERK1/2 signaling via formyl peptide receptors, leading to ROS production and microvascular leakage. Therefore, our study reveals that Bam32-mediated changes in WKYMVm-induced microvascular hyperpermeability are largely due to Bam32-dependent, ERK1/2-involving production of ROS by the recruited neutrophils.

The proinflammatory effects of WKYMVm have been reported previously [328,329,381]. We show in this study that this formyl peptide also elevated microvascular leakage only in WT mice. Deficiency of Bam32 significantly lowered hyperpermeability in WKYMVm-treated mouse microvasculature, especially at late time points (after 40 min), but increased microvascular hyperpermeability at 15–25 min in chemokine CXCL2-treated mouse microvasculature. The role of Bam32 in WKYMVm-induced vascular hyperpermeability was in the recruited neutrophils because a significantly lower number of emigrated neutrophils was found simultaneously in the same segment of venule where the microvascular permeability was determined. The neutrophil-intrinsic function of Bam32 is supported by the results from bone marrow chimeric mice with WKYMVm-superfused cremasteric vasculature. Chimeric mice with Bam32-deficient neutrophils, regardless of their endothelial background, had decreased microvascular hyperpermeability as well as the number of emigrated neutrophils, strongly indicating that the role of Bam32 in neutrophils is sufficient to significantly impact WKYMVm-induced venule barrier functions in response to WKYMVm. Interestingly, a higher level of leakage in WT→Bam32$^{-/-}$ mice than in WT→WT mice was noticeable at 15 min time point after superfusion with WKYMVm. Although this difference was transient and only appears in chimeric mice with WT neutrophils, it suggests the

possibility that a subordinate Bam32-mediated mechanism for regulating microvascular leakage may exist in endothelial cells.

Neutrophil-dependent local production of pro-inflammatory mediators, such as oxidants [333], impairs vascular barrier function through various mechanisms. Previous studies reported that ROS increased the phosphorylation of catenin by activating tyrosine kinases in endothelial cells. This phosphorylation facilitates the internalization of VE-cadherin, ending up with the opening of adherens junctions [390]. Moreover, ROS regulate actin rearrangement in endothelial cells through Rac1 activation to increase microvascular permeability [391]. In addition, ROS damage tight junctions of endothelium and increase the leakage by inducing redistribution of the transmembrane proteins [132]. In our study, the production of intracellular and extracellular ROS in WKYMVm-stimulated neutrophils was impaired by the deficiency of Bam32. Our finding that NADPH oxidase inhibitor DPI and hydrogen peroxide metabolizing enzyme catalase blocked WKYMVm-induced increases in microvascular permeability suggests a critical functional role of Bam32-dependent ROS production in WKYMVm-induced increases in mouse microvascular barrier functions. In contrast, chemokine CXCL2 failed to induce differences in ROS generation between WT neutrophils and Bam32$^{-/-}$ neutrophils even after TNFα priming, indicating that deficiency of Bam32 increases CXCL2-induced microvascular hyperpermeability through a completely ROS-independent mechanism.

As prerequisite events to the local ROS production of recruiting neutrophils, neutrophil adhesion and emigration trigger microvascular leakage through physical interactions with the endothelium [331,332]. However, our data show that the role of Bam32 in mediating microvascular leakage may be due to Bam32-dependent production of ROS in neutrophils, rather than Bam32-dependent emigration. This is because, after inhibition of ROS production by DPI in WKYMVm-treated mice, the persisting significant gap in the numbers of adherent and emigrated neutrophils was insufficient to induce Bam32-dependent differences in microvascular hyperpermeability. This uncoupling of neutrophil adhesion and emigration to microvascular leakage was also reported in previous studies. Leukotriene B_4-induced transmigrating neutrophils infiltrated into alveolar spaces

of human lungs without increasing microvascular permeability [337]. The formation of the endothelial dome structure, also known as the transmigratory cup, encapsulated the transmigrating neutrophils and minimized the microvascular leakage [32,38]. Therefore, our study suggests that Bam32-dependent increases in WKYMVm-induced microvascular permeability are attributed to the alteration in Bam32-dependent production of ROS in recruiting neutrophils.

Rac1 is mainly referred in regulating the polarization and directionality in neutrophil chemotaxis [277,279], but also involved in NADPH oxidase-mediated ROS generation in neutrophils [392]. Given the possible involvement of Rac1 in WKYMVm-induced neutrophil ROS production, we determined the Rac1 activation in WKYMVm-stimulated neutrophils. Interestingly, our results show that Bam32 was required in WKYMVm-induced Rac1 activation. Although our study suggests a role for Bam32 in NADPH oxidase-mediated ROS generation, this Bam32-dependent functional connection between Rac1 activation and ROS generation may require further verification.

Bam32, albeit well-established as an adaptor molecule downstream of the PI3K signaling pathway, plays a role in mediating MAPK signals in immune cells. Bam32 in B cells regulates cell proliferation and T cell-independent antigen responses through phosphorylation of ERK and JNK [260,263]. Moreover, Bam32 in T cells is important for optimal TCR-mediated ERK1/2 activation [272]. In this study, we revealed a novel role of Bam32 in modulating ERK1/2 signaling without influencing p38 and JNK in WKYMVm-stimulated neutrophils. We further demonstrated that signaling via ERK1/2 is required for WKYMVm-induced ROS production and subsequent microvascular leakage based on our experiments using BVD-523, an ATP-competitive and reversible covalent inhibitor of ERK1/2 kinases, and PD98059, a highly selective inhibitor of MEK1 activation in the MAPK signaling cascade. The application of these two ERK1/2 inhibitors showed similar trend in impairing neutrophil ROS generation and microvascular hyperpermeability in response to WKYMVm and supported the role of ERK1/2 signaling in Bam32-dependent ROS generation and microvascular permeability changes. It is worth noting that the data of BVD-523 inhibition should be taken cautiously because of the possibly nonspecific inhibition of PMA-

induced, protein kinase C-involving neutrophil ROS production unraveled in this study.

A previous study found that the activation of ERK1/2 signaling results in conformational changes in $p47^{phox}$, an important component of NADPH oxidase for ROS production [393], indicating a potential mechanism linking ERK with ROS production. Reciprocally, the production of ROS modulates ERK1/2 phosphorylation [394,395], suggesting that the low production of ROS in Bam32-deficient neutrophils may, in turn, contribute to the reduced ERK1/2 phosphorylation in WKYMVm-stimulated neutrophils. Given this bidirectional cross-talk between ERK activation and ROS production, further explorations will be needed to reveal the interactions between Bam32-dependent ROS production and Bam32-dependent ERK1/2 signaling in the process of microvascular leakage.

Bam32 is required for WKYMVm-induced hyperpermeability in mouse cremasteric post-capillary venule through enhancing the generation of extracellular and intracellular ROS. This role of Bam32 in neutrophils for microvascular hyperpermeability is not functionally linked to neutrophil adhesion and emigration. Bam32-mediated ERK1/2 signaling is involved in the production of ROS induced by WKYMVm and the inhibition of either ERK1/2 or ROS can block WKYMVm-induced hyperpermeability. Our study provides a mechanistic insight into the role of Bam32 in neutrophils for regulating microvascular leakage in acute inflammation where formyl peptide receptors are involved.

CHAPTER SIX

GENERAL DISCUSSION AND CONCLUSIONS

6.1 General discussion

The roles of PI3K adaptor Bam32 in immunity have been revealed gradually during the past twenty years since it was firstly discovered in mouse B lymphocytes in 2000. Herein, for the very first time, we explored the roles of Bam32, as well as its upstream PI3K isoforms, in regulating mouse neutrophil recruitment and microvascular hyperpermeability induced by different chemoattractants in acute inflammation through using intravital microscopy and the related biomedical approaches *in vivo* and *in vitro*. We further unveiled that Bam32 suppressed CXCL2-induced neutrophil recruitment through reducing the activation of the small GTPase Rap1. Moreover, Bam32 and its upstream PI3Kγ regulated WKYMVm-induced microvascular leakage via the local generation of ROS by the recruiting neutrophils. The observations we obtained from this study, together with the in-depth Bam32-dependent mechanisms, strongly suggest that Bam32 plays pivotal roles in various neutrophil responses in acute inflammation.

6.1.1 The stimulus-dependent dual role of Bam32 in neutrophil recruitment

Throughout this study, we used four chemoattractants with different mechanisms, which are the CXC chemokine CXCL2 that binds to CXC receptors [396], the formyl peptide receptor agonist WKYMVm that binds to formyl peptide receptors [306], the cytokine TNFα that binds to TNF receptors [397], and the glycogen from oyster that binds to Toll-like receptors [398]. Among these stimuli, we mainly focused on CXCL2 and WKYMVm, the classical intermediary chemoattractant and end-target chemoattractant, respectively. Surprisingly, we found that deficiency of Bam32 resulted in completely opposite responses induced by these two chemoattractants. The deficiency of Bam32 enhanced CXCL2-induced neutrophil transmigration but reduced WKYMVm-induced neutrophil transmigration on mouse cremaster muscle, suggesting that Bam32 may play a stimulus-dependent dual role in regulating neutrophil recruitment. This stimulus-dependent dual role of

Bam32 may be owing to the activation of different signaling pathways in neutrophils, given that CXCRs preferentially activate the PI3K signaling pathway and FPRs preferentially activate the MAPK signaling pathway [36]. Similar to the neutrophil recruitment induced by WKYMVm, the deficiency of Bam32 suppressed neutrophil emigration in mouse peritonitis induced by glycogen from oyster, indicating that Bam32 also functions in modulating signals downstream of TLRs. Moreover, the deficiency of Bam32 only reduced the rolling velocity and rolling flux without influencing neutrophil adhesion and emigration induced by TNFα. This suggests a possible role of Bam32 in regulating TNFα-induced leukocyte slow rolling. Meanwhile, we cannot exclude the possibility of the role of Bam32 in TNFα-induced neutrophil recruitment because we only observed the net consequences of the massive release of chemokines triggered by TNFα. It is entirely possible that the function of Bam32 in TNFα-treated mice were covered up by multiple opposite responses induced by each individual cytokine. Therefore, our study for the first time reveals that Bam32 functions widely in neutrophil inflammatory responses and the role of Bam32 is stimulus dependent.

6.1.2 The net function of Bam32 in neutrophil-mediated innate immunity

The supportive role of PI3K signaling pathway in innate immunity has been widely accepted, however, the role of Bam32, one of the PI3K downstream adaptors, in innate immunity is still an open question. PI3Kγ, as shown in this study and previous studies, is required in both intermediary and end-target chemoattractant-induced neutrophil recruitment [228,399,400]. After the arrival of neutrophils at the inflammatory site, PI3Kγ is again required in the production of ROS from neutrophils to clear the pathogens and change microvascular permeability. In contrast, the role of Bam32 in neutrophils is more complicated. Firstly, the role of Bam32 in intermediary chemoattractant CXCL2-induced neutrophil recruitment is suppressive. This is for the first time revealed and verified in different tissues both *in vivo* and *in vitro* by our study. This suppressive role of Bam32 regulate CXCL2-induced neutrophil recruitment through modulating the activation of small GTPase Rap1 and further changing the phosphorylation of a PI3K effector Akt. Secondly, the role of Bam32 in end-target chemoattractant WKYMVm-induced neutrophil recruitment is supportive because of our finding that deficiency of Bam32 impairs WKYMVm-induced

neutrophil recruitment in mouse cremaster muscle. In most cases, the acute inflammation is triggered by a combination of multiple stimuli and the transition of stimulation with chemoattractants from intermediary to end-target is consecutive and gradually varied. Therefore, the role of Bam32 in neutrophil recruitment may also switch from suppressive at the intermediary chemokine-dominated early stage to supportive at the end-target chemokine-dominated later stage. Then, the net function of Bam32 is dependent on the proportion of stimuli that bind to the surface receptors on neutrophils. This conclusion can also explain our observations of the null effect of Bam32 deficiency on TNFα-induced neutrophil recruitment because TNFα triggers massive release of various stimuli from endothelial cells.

In addition, when neutrophils arrive at the inflammatory site approaching end-target chemoattractant-dominated environment, the role of Bam32 in neutrophils is supportive, proven by our study of WKYMVm-induced production of ROS in neutrophils without Bam32. Our study shows that Bam32 is critically required in the production of ROS in neutrophils and assists in increasing microvascular hyperpermeability, which may further facilitate the transmigration of subsequent leukocytes, through the local generation of ROS in adherent neutrophils. Therefore, Bam32 plays a supportive role in this scenario.

6.1.3 The Bam32-mediated signaling pathways

Upon stimulation with chemoattractants, several Bam32-mediated signaling pathways downstream of the surface receptors in neutrophils are activated. Many previous studies reported that Bam32 functions as a PI3K downstream adaptor by binding both PI3K substrates PI(3,4)P2 and PI(3,4,5)P3 in various types of leukocytes [257,260,348]. In this study, we reveal such a similar role of Bam32 downstream of PI3K signaling pathway in neutrophils. Meanwhile, we also demonstrate an interaction between Bam32 and the PI3K effector Akt through small GTPase Rap1. Bam32 is required in the activation of its downstream small GTPase Rap1, further suppressing the phosphorylation of Akt in neutrophils. When Bam32 is absent, the Rap1-dependent suppression on the activation of Akt is removed. As a consequence, enhanced neutrophil recruitment was observed in the cremaster muscle of Bam32-deficient mice in our study because of the up-regulated Akt phosphorylation.

Beyond the well-known role downstream of PI3K signaling pathway, Bam32 also plays a role in MAPK signaling pathway. Previous studies showed that Bam32 regulates B cell proliferation and antigen responses through activation of p38 and participates in TCR-mediated phosphorylation of ERK1/2 [272]. In our study, we show that Bam32 regulates WKYMVm-induced ROS generation through the phosphorylation of ERK1/2 but not p38 or JNK. Nevertheless, our study cannot exclude the possibility that the Bam32-dependent ROS in turn enhance the phosphorylation of ERK1/2.

6.1.4 A potential role of Bam32 in endothelial cells

In endothelial cells, the expression of Bam32 is not as high as that in immune cells but still quantifiable like in other tissues [257]. Our study mainly focuses on the role of Bam32 in neutrophils, however, a possible role of Bam32 in endothelial cells is indicated by some clues in our study and should not be neglected. Firstly, given that TNFα triggers endothelial cells to express surface adhesion molecules and to release high levels of a number of chemoattractants [126,401], our results show that the deficiency of Bam32 slows the intraluminal rolling velocity of leukocytes in mouse cremaster muscle after 4-h treatment with TNFα, indicating a role of Bam32 in regulating the expression of adhesion molecules or the release of chemoattractants in endothelial cells. Secondly, our results also show that the microvascular leakage in WT→Bam32$^{-/-}$ chimeric mice is significantly higher than that in WT→WT chimeric control mice at 15 min after superfusion with WKYMVm, suggesting a defect in endothelial integrity caused by the deficiency of Bam32. However, this defect will only show up when normal circulatory neutrophils are available, which is evidenced by the fact that the microvascular hyperpermeability in Bam32$^{-/-}$→WT chimeric mice is similar to that in Bam32$^{-/-}$→Bam32$^{-/-}$ chimeric control mice after superfusion with WKYMVm. Therefore, Bam32 in endothelial cells may also be involved in regulating neutrophil recruitment and endothelial integrity, albeit Bam32 in neutrophils plays a dominant role.

6.1.5 Possible involvement of other cell types in neutrophil recruitment

Throughout this thesis, I focused on neutrophil recruitment, microvascular hyperpermeability, and the signaling events during the interactions between neutrophils and endothelial cells in the microvasculature. It is worth noting that other cell types in this microenvironment may also contribute to the regulation of neutrophil recruitment.

Mast cells, residing widely in most tissues, respond to stimuli *via* pattern recognition receptors TLR and IL-1 receptor-like 1 [402,403]. The activation of mast cells, triggered by damaged tissue components, microbial products or the binding of IgE and Fcε receptors, results in mast cell degranulation that releases histamine, prostaglandins, leukotrienes, and thromboxane [404]. Albeit indirectly, these inflammatory mediators increase neutrophil extravasation by mobilizing P-selectin to the luminal endothelial surface and facilitating the firm adhesion for neutrophils [405]. Moreover, the mediators also increase the tyrosine phosphorylation of VE-cadherin in the junctions of endothelial cells, thus elevate the microvascular permeability [406].

Another type of resident immune cell is the perivascular macrophage that resides outside the basement membrane. Macrophages promote neutrophil recruitment through releasing chemokines CXCL1, CXCL2, and CCL3 [407]. Because of the discontinuous association of macrophages to the basement membrane, the chemokines released from macrophages are not evenly distributed on the endothelial luminal surface but deposited in scatters, causing the patchy arrest of neutrophils to the post-capillary venule wall.

Beyond the immune cells, pericytes, a cell type that constitutes the endothelial barrier together with endothelial cells, also regulate neutrophil recruitment and microvascular permeability. First, pericytes express TLR and cytokine receptors to sense the tissue damage and release inflammatory mediators to promote neutrophil firm adhesion [408,409]. Then, the adherent neutrophils will search for the low expression regions of laminin-8, laminin-10, and collagen type IV, which are located at the gaps between adjacent pericytes, for transendothelial migration. The detailed communications between neutrophils and pericytes during transmigration are still unknown, but it was believed that LTB_4 released by the previously transmigrated neutrophils was the driven force to keep the subsequent transmigration of neutrophils at the pericyte gaps [410].

Second, pericytes express ICAM-1 and release chemokines to facilitate the intraluminal crawling of neutrophils [409]. Third, the gaps between pericytes may enlarge in response to stimulations with TNFα or IL-1β. This might be because TNFα or IL-1β triggers pericytes to contract through the small GTPase-mediated actin cytoskeleton rearrangement [409,411].

Lastly, platelets are also known to be critical in promoting neutrophil recruitment. They function like "little binders" between endothelial cells and neutrophils. On one side, platelets bind endothelial cells through the surface $\alpha_{IIb}\beta_3$ integrins; on the other side, this integrin binds fibrinogen, which further binds Mac-1 on neutrophils, eventually forming the firm adhesion of neutrophil-platelet complexes to the endothelium [412–415]. Moreover, platelet CD40 could bind to neutrophil CD40L, in turn activating both involved platelets and neutrophils [416]. In addition to the physical contacts, the activated platelets induce neutrophil extravasation, apoptosis, and NETosis through the production of growth factors, chemokines, and cytokines [417,418].

6.2 Conclusions

- PI3Kγ is required in WKYMVm-induced but not CXCL2-induced microvascular hyperpermeability in mice.
- Neutrophils but not endothelial cells are the dominant triggering cells that are important for the WKYMVm-induced, PI3Kγ-specific microvascular hyperpermeability.
- PI3Kγ-dependent ROS generation in neutrophils results in WKYMVm-induced, PI3Kγ-specific microvascular hyperpermeability in mice.

- Bam32 suppresses CXCL2-induced neutrophil recruitment in cremaster muscle and peritoneum in mice.
- Bam32 suppresses CXCL2-induced neutrophil chemotaxis *in vitro*.
- Bam32 and the activation of Rac1, are required in CXCL2-induced activation of Rap1.
- Bam32-dependent Rap1 activation regulates CXCL2-induced neutrophil recruitment through modulating the phosphorylation of Akt in mice.

- Bam32 is required in WKYMVm-induced microvascular hyperpermeability in mice.
- Bam32 enhances WKYMVm-induced neutrophil recruitment in the cremaster muscle in mice.
- Bam32-dependent ROS generation in neutrophils results in WKYMVm-induced, Bam32-specific microvascular hyperpermeability in mice.
- ERK1/2 but not p38 or JNK MAPK is involved in WKYMVm-induced, Bam32-specific ROS generation and microvascular hyperpermeability in mice.

6.3 Limitations of this study

Firstly, we only explored the roles of two isoforms of Class I PI3Ks (PI3Kγ and PI3Kδ) and revealed that PI3Kγ is critical in the production of neutrophil ROS which regulates microvascular permeability. We could not exclude the possibility that other Class I PI3Ks (PI3Kα and PI3Kβ), Class II PI3K (PI3KC2α, PI3KC2β, PI3KC2γ) and Class III PI3K may also influence WKYMVm-induced ROS production in neutrophils. It would be of great value to use specific PI3K isoform inhibitors and evaluate their roles in the production of different types of ROS *in vitro*. In the future studies, we are planning to use different inhibitors for Class I PI3Ks (Ia: PI3Kα, PI3Kβ and PI3Kδ; Ib: PI3Kγ), and inhibitors for Class II PI3Ks and Class III PI3Ks to scrutinize their roles for the WKYMVm-induced production of superoxide and hydrogen peroxide.

Secondly, we only determined the production of intracellular superoxide and extracellular hydrogen peroxide *in vitro*. Given that hydrogen peroxide is the final product of most reactive oxygen species pathways, our study may not identify the generation of specific type(s) of ROS that are dependent on PI3Kγ or Bam32. Further studies on the corresponding regulatory and catalytic subunits of PI3Kγ can be done to illustrate the underlying mechanisms of PI3K-dependent and Bam32-dependent ROS production. Moreover, the specific type(s) of ROS that are dependent on PI3Kγ or Bam32 are also worth of exploring.

Thirdly, although we reported that Bam32 regulates CXCL2-induced neutrophil recruitment through Rap1, we cannot exclude the possibility of the involvement of Rac in Bam32-regulated neutrophil recruitment. Rac1 is known functioning in the formation of leading-edge and uropod events *via* regulating Rho and myosin activation in both human and mouse neutrophils [279]. Moreover, Rac1 also modulates the gradient detection of chemokines, which acts as the "compass" for directional chemotaxis in neutrophils [277,375]. On the other hand, Rac2, another member of Rac family, regulates the superoxide production in neutrophils and modulates neutrophil recruitment via integrin-mediated spreading and actin assembly [374]. In our study, some results of Rac1 are found inconsistent with previous studies and the lack of evidence on Rac2 in CXCL2-induced neutrophil recruitment implies that more explorations will be required to address the possible involvement of Rac in Bam32-regulated neutrophil recruitment in mice.

6.4 Future prospective

The roles of Bam32, one of downstream adaptor molecules of PI3K signaling pathway, have been established in adaptive immunity since Bam32 was first discovered in 2000. In this thesis, I revealed unreported roles of Bam32 in neutrophil recruitment and neutrophil ROS generation through stimulus-specific mechanisms during acute inflammation. Given that the importance of neutrophils in the pathogenesis of chronic diseases was reported by an increasing number of studies, Bam32, an adaptor molecule neglected by researchers for many years, may be a good candidate of therapeutic target for preventing and treating many different chronic diseases.

6.4.1 Cardiovascular diseases

Neutrophils are known to be involved in different stages of atherosclerosis. Although the comprehensive mechanisms are still poorly identified, it was proposed that the increase in local oxidative stress, possibly posed by recruited neutrophils in the lesions, may disrupt the normal functions of the endothelium, thus increase the sizes of lesions and the possibility of intraplaque hemorrhage [50–53]. In my study, Bam32 regulates chemokine-induced neutrophil recruitment and formyl peptide receptor agonist-induced ROS production from neutrophils, suggesting that Bam32 might be involved in the initial, development, and rupture stages of atherosclerosis. However, it should be noted that albeit neutrophil surface markers Ly-6G and CD66b were found in the atherosclerotic lesions, no direct evidence so far showed the actual abundance of neutrophils in the lesions because it is entirely possible that those neutrophil surface markers could drop off from the neutrophil membrane and accidentally engulfed by macrophages that may later transform to foam cells in the atherosclerotic lesions. The fact that depletion of neutrophils by Ly-6G antibodies decreased the lesion sizes may slightly increase the credibility of neutrophils in contributing to the development of lesions, but this is still debatable because of the off-target effects of Ly-6G antibodies. In other scenarios, ROS also increase systemic blood pressure by consuming nitric oxides and producing peroxynitrites, but these ROS are mainly from endothelial cells, in which Bam32 seems to play a minor role due to its comparatively low level expression [161,164,165].

6.4.2 Metabolic disorders

The status of sterile inflammation is widely accepted as the initial stage of many metabolic disorders, such as obesity and diabetes. Neutrophils, albeit having a short half-life, decrease the energy expenditure and cause obesity through suppressing β-oxidation of fatty acids and releasing elastase which blocks insulin functions by depleting insulin receptor substrate 1 [58,59]. As a result, the increased glucose level further promotes neutrophil NETosis which deteriorates the complications of diabetes, such as wound healing and tissue repair [58]. My present study not only showed the role of Bam32 in mediating neutrophil recruitment and chemotaxis in tissues, but also found that Bam32-deficient mice had significantly higher average body weights than the control WT mice (data not shown). Moreover, the deficiency of Bam32 changed the activation of PI3K effector Akt, which is a critical modulator in regulating body metabolism. These clues hint that Bam32 might have its unique role in metabolism and in certain metabolic disorders.

6.4.3 CNS diseases

In the epilepsy mouse model, increased neutrophil recruitment and vascular permeability in the brain were noticed accompanying with the acute symptoms, which would be alleviated by the depletion of neutrophils [56,57]. Considering that Bam32 regulates neutrophil recruitment as well as microvascular hyperpermeability shown in my research, it will be of great interest to explore if Bam32 could be a potential target for preventing the acute and recurrent symptoms in epilepsy, especially when leukocyte-derived ROS are involved. In Alzheimer's disease, the accumulation of amyloid β increases LFA-mediated neutrophil recruitment, stimulates lymphocyte-derived ROS generation, and thereafter induces neuron death through lipid peroxidation [54,55,159]. As per our observations in the present study, Bam32 regulates neutrophil adhesion and neutrophil ROS generation, implying that inhibiting the functions of Bam32 might be an alternative way to block the notorious effects of amyloid β.

Although the potential connections of Bam32 to the CNS diseases seem tempting, the

expression of Bam32 in CNS cell types needs to be profiled. Currently, only the expression of Bam32 mRNA has been determined in the brain tissue and is much lower than that in leukocytes. It would be surprising if such low expression of Bam32 may play a major role in most CNS cell types but it would be totally expectable that abundant expression of Bam32 in microglia, a type of resident macrophages in the brain, may contribute to many CNS diseases.

6.4.4 Cancer

The role of neutrophils in long-term tumorigenesis, albeit interesting, remains largely uninvestigated. To date, neutrophils are believed to promote the tumor initiation *via* producing ROS to interrupt the cellular DNA and to facilitate the tumor metastasis and angiogenesis *via* releasing MMP9 and elastase [65–68]. In my study, Bam32 showed its critical role in neutrophil ROS generation, suggesting that a possible connection might be made towards the tumorigenesis. However, it should be clarified that my study used WKYMVm which mimics the effects of PAMPs to show the importance of Bam32 in neutrophil ROS generation. More tumor-related stimulants could be considered in future studies. In addition, since neutrophils have four types of granules that contain distinctive enzymes to function separately, a detailed profile of Bam32 in the production and releasing of the granule enzymes in neutrophils will also be of great interest.

References

1. Summers C, Rankin SM, Condliffe AM, Singh N, Peters AM, Chilvers ER: **Neutrophil kinetics in health and disease**. *Trends Immunol* 2010, **31**:318–324.
2. Peters AM: **Just how big is the pulmonary granulocyte pool?** *Clin Sci* 1998, **94**:7–19.
3. Myal Y, Del Bigio MR, Rhodes RH: **Age-related differences in 1p and 19q deletions in oligodendrogliomas**. *BMC Clin Pathol* 2003, **3**:1–9.
4. Mestas J, Hughes CCW: **Of Mice and Not Men: Differences between Mouse and Human Immunology**. *J Immunol* 2004, **172**:2731–2738.
5. Galli SJ, Borregaard N, Wynn TA: **Phenotypic and functional plasticity of cells of innate immunity: Macrophages, mast cells and neutrophils**. *Nat Immunol* 2011, **12**:1035–1044.
6. Pillay J, Den Braber I, Vrisekoop N, Kwast LM, De Boer RJ, Borghans JAM, Tesselaar K, Koenderman L: **In vivo labeling with 2H2O reveals a human neutrophil lifespan of 5.4 days**. *Blood* 2010, **116**:625–627.
7. Colotta F, Re F, Polentarutti N, Sozzani S, Mantovani A: **Modulation of granulocyte survival and programmed cell death by cytokines and bacterial products**. *Blood* 1992, **80**:2012–2020.
8. Faurschou M, Borregaard N: **Neutrophil granules and secretory vesicles in inflammation**. *Microbes Infect* 2003, **5**:1317–1327.
9. Borregaard N: **Neutrophils, from Marrow to Microbes**. *Immunity* 2010, **33**:657–670.
10. Tsuda Y, Takahashi H, Kobayashi M, Hanafusa T, Herndon DN, Suzuki F: **Three different neutrophil subsets exhibited in mice with different susceptibilities to infection by methicillin-resistant Staphylococcus aureus**. *Immunity* 2004, **21**:215–226.
11. Johnston B, Burns AR, Suematsu M, Issekutz TB, Woodman RC, Kubes P: **Chronic inflammation upregulates chemokine receptors and induces neutrophil migration to monocyte chemoattractant protein-1**. *J Clin Invest* 1999, **103**:1269–1276.
12. Häger M, Cowland JB, Borregaard N: **Neutrophil granules in health and disease**. *J Intern Med* 2010, **268**:25–34.

13. Phillipson M, Kubes P: **The neutrophil in vascular inflammation**. *Nat Med* 2011, **17**:1381–1390.

14. Papayannopoulos V, Zychlinsky A: **NETs: a new strategy for using old weapons**. *Trends Immunol* 2009, **30**:513–521.

15. Hong C, Kidani Y, A-Gonzalez N, Phung T, Ito A, Rong X, Ericson K, Mikkola H, Beaven SW, Miller LS, et al.: **Coordinate regulation of neutrophil homeostasis by liver X receptors in mice**. *J Clin Invest* 2012, **122**:337–347.

16. Shi J, Gilbert GE, Kokubo Y, Ohashi T: **Role of the liver in regulating numbers of circulating neutrophils**. *Blood* 2001, **98**:1226–1230.

17. Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, Weinrauch Y, Zychlinsky A: **Neutrophil Extracellular Traps Kill Bacteria**. *Science* 2004, **303**:1532–1535.

18. McDonald B, Urrutia R, Yipp BG, Jenne CN, Kubes P: **Intravascular neutrophil extracellular traps capture bacteria from the bloodstream during sepsis**. *Cell Host Microbe* 2012, **12**:324–333.

19. Yipp BG, Petri B, Salina D, Jenne CN, Scott BNV, Zbytnuik LD, Pittman K, Asaduzzaman M, Wu K, Meijndert HC, et al.: **Infection-induced NETosis is a dynamic process involving neutrophil multitasking in vivo**. *Nat Med* 2012, **18**:1386–1393.

20. Eash KJ, Means JM, White DW, Link DC: **CXCR4 is a key regulator of neutrophil release from the bone marrow under basal and stress granulopoiesis conditions**. *Blood* 2009, **113**:4711–4719.

21. Kolaczkowska E, Kubes P: **Neutrophil recruitment and function in health and inflammation**. *Nat Rev Immunol* 2013, **13**:159–175.

22. Ley K, Laudanna C, Cybulsky MI, Nourshargh S: **Getting to the site of inflammation: The leukocyte adhesion cascade updated**. *Nat Rev Immunol* 2007, **7**:678–689.

23. Petri B, Phillipson M, Kubes P: **The Physiology of Leukocyte Recruitment: An In Vivo Perspective**. *J Immunol* 2008, **180**:6439–6446.

24. Zarbock A, Ley K, McEver RP, Hidalgo A: **Leukocyte ligands for endothelial selectins: Specialized glycoconjugates that mediate rolling and signaling under flow**. *Blood* 2011, **118**:6743–6751.

25. Wisse E, de Zanger RB, Charels K, van der Smissen P, McCuskey RS: **The liver sieve: Considerations concerning the structure and function of endothelial fenestrae, the sinusoidal wall and the space of disse**. *Hepatology* 1985, **5**:683–692.

26. Wong J, Johnston B, Lee SS, Bullard DC, Smith CW, Beaudet AL, Kubes P: **A minimal role for selectins in the recruitment of leukocytes into the inflamed liver microvasculature**. *J Clin Invest* 1997, **99**:2782–2790.

27. McDonald B, McAvoy EF, Lam F, Gill V, De La Motte C, Savani RC, Kubes P: **Interaction of CD44 and hyaluronan is the dominant mechanism for neutrophil sequestration in inflamed liver sinusoids**. *J Exp Med* 2008, **205**:915–927.

28. Dewey CF, Bussolari SR, Gimbrone MA, Davies PF: **The dynamic response of vascular endothelial cells to fluid shear stress**. *J Biomech Eng* 1981, **103**:177–185.

29. Burns AR, Bowden RA, MacDonell SD, Walker DC, Odebunmi TO, Donnachie EM, Simon SI, Entman ML, Smith CW: **Analysis of tight junctions during neutrophil transendothelial migration**. *J Cell Sci* 2000, **113**:45–57.

30. Woodfin A, Voisin MB, Beyrau M, Colom B, Caille D, Diapouli FM, Nash GB, Chavakis T, Albelda SM, Rainger GE, et al.: **The junctional adhesion molecule JAM-C regulates polarized transendothelial migration of neutrophils in vivo**. *Nat Immunol* 2011, **12**:761–769.

31. Petri B, Kaur J, Long EM, Li H, Parsons SA, Butz S, Phillipson M, Vestweber D, Patel KD, Robbins SM, et al.: **Endothelial LSP1 is involved in endothelial dome formation, minimizing vascular permeability changes during neutrophil transmigration in vivo**. *Blood* 2011, **117**:942–952.

32. Phillipson M, Kaur J, Colarusso P, Ballantyne CM, Kubes P: **Endothelial domes encapsulate adherent neutrophils and minimize increases in vascular permeability in**

paracellular and transcellular emigration. *PLoS One* 2008, **3**.

33. Parsons SA, Sharma R, Roccamatisi DL, Zhang H, Petri B, Kubes P, Colarusso P, Patel KD: **Endothelial paxillin and focal adhesion kinase (FAK) play a critical role in neutrophil transmigration**. *Eur J Immunol* 2012, **42**:436–446.
34. Betsuyaku T, Shipley JM, Liu Z, Senior RM: **Neutrophil emigration in the lungs, peritoneum, and skin does not require gelatinase B**. *Am J Respir Cell Mol Biol* 1999, **20**:1303–1309.
35. Heit B, Robbins SM, Downey CM, Guan Z, Colarusso P, Miller JB, Jirik FR, Kubes P: **PTEN functions to "prioritize" chemotactic cues and prevent "distraction" in migrating neutrophils**. *Nat Immunol* 2008, **9**:743–752.
36. Heit B, Tavener S, Raharjo E, Kubes P: **An intracellular signaling hierarchy determines direction of migration in opposing chemotactic gradients**. *J Cell Biol* 2002, **159**:91–102.
37. Nourshargh S, Renshaw SA, Imhof BA: **Reverse Migration of Neutrophils: Where, When, How, and Why?** *Trends Immunol* 2016, **37**:273–286.
38. Phillipson M, Heit B, Colarusso P, Liu L, Ballantyne CM, Kubes P: **Intraluminal crawling of neutrophils to emigration sites: A molecularly distinct process from adhesion in the recruitment cascade**. *J Exp Med* 2006, **203**:2569–2575.
39. Micali G, Endres RG: **Bacterial chemotaxis: Information processing, thermodynamics, and behavior**. *Curr Opin Microbiol* 2016, **30**:8–15.
40. Nichols JME, Veltman D, Kay RR: **Chemotaxis of a model organism: Progress with Dictyostelium**. *Curr Opin Cell Biol* 2015, **36**:7–12.
41. Mortimer D, Fothergill T, Pujic Z, Richards LJ, Goodhill GJ: **Growth cone chemotaxis**. *Trends Neurosci* 2008, **31**:90–98.
42. Elks PM, Van Eeden FJ, Dixon G, Wang X, Reyes-Aldasoro CC, Ingham PW, Whyte MKB, Walmsley SR, Renshaw SA: **Activation of hypoxia-inducible factor-1α (hif-1α) delays inflammation resolution by reducing neutrophil apoptosis and reverse migration in a zebrafish inflammation model**. *Blood* 2011, **118**:712–722.

43. Deng Q, Huttenlocher A: **Leukocyte migration from a fish eye's view**. *J Cell Sci* 2012, **125**:3949–3956.

44. Thompson AAR, Elks PM, Marriott HM, Eamsamarng S, Higgins KR, Lewis A, Williams L, Parmar S, Shaw G, McGrath EE, et al.: **Hypoxia-inducible factor 2a regulates key neutrophil functions in humans, mice, and zebrafish**. *Blood* 2014, **123**:366–376.

45. Colom B, Bodkin J V., Beyrau M, Woodfin A, Ody C, Rourke C, Chavakis T, Brohi K, Imhof BA, Nourshargh S: **Leukotriene B4-Neutrophil Elastase Axis Drives Neutrophil Reverse Transendothelial Cell Migration InVivo**. *Immunity* 2015, **42**:1075–1086.

46. Duffy D, Perrin H, Abadie V, Benhabiles N, Boissonnas A, Liard C, Descours B, Reboulleau D, Bonduelle O, Verrier B, et al.: **Neutrophils Transport Antigen from the Dermis to the Bone Marrow, Initiating a Source of Memory CD8+ T Cells**. *Immunity* 2012, **37**:917–929.

47. McDonald B, Pittman K, Menezes GB, Hirota SA, Slaba I, Waterhouse CCM, Beck PL, Muruve DA, Kubes P: **Intravascular danger signals guide neutrophils to sites of sterile inflammation**. *Science* 2010, **330**:362–366.

48. Chen GY, Nuñez G: **Sterile inflammation: Sensing and reacting to damage**. *Nat Rev Immunol* 2010, **10**:826–837.

49. Döring Y, Drechsler M, Soehnlein O, Weber C: **Neutrophils in atherosclerosis: From mice to man**. *Arterioscler Thromb Vasc Biol* 2015, **35**:288–295.

50. Rotzius P, Thams S, Soehnlein O, Kenne E, Tseng CN, Björkström NK, Malmberg KJ, Lindbom L, Eriksson EE: **Distinct infiltration of neutrophils in lesion shoulders in ApoE -/- mice**. *Am J Pathol* 2010, **177**:493–500.

51. Drechsler M, Megens RTA, Van Zandvoort M, Weber C, Soehnlein O: **Hyperlipidemia-triggered neutrophilia promotes early atherosclerosis**. *Circulation* 2010, **122**:1837–1845.

52. Ionita MG, Van Den Borne P, Catanzariti LM, Moll FL, De Vries JPPM, Pasterkamp G, Vink A, De Kleijn DPV: **High neutrophil numbers in human carotid atherosclerotic plaques are associated with characteristics of rupture-prone lesions**. *Arterioscler Thromb Vasc*

Biol 2010, **30**:1842–1848.

53. Moreno JA, Ortega-Gómez A, Delbosc S, Beaufort N, Sorbets E, Louedec L, Esposito-Farèse M, Tubach F, Nicoletti A, Steg PG, et al.: **In vitro and in vivo evidence for the role of elastase shedding of CD163 in human atherothrombosis**. *Eur Heart J* 2012, **33**:252–263.

54. Baik SH, Cha MY, Hyun YM, Cho H, Hamza B, Kim DK, Han SH, Choi H, Kim KH, Moon M, et al.: **Migration of neutrophils targeting amyloid plaques in Alzheimer's disease mouse model**. *Neurobiol Aging* 2014, **35**:1286–1292.

55. Zenaro E, Pietronigro E, Bianca V Della, Piacentino G, Marongiu L, Budui S, Turano E, Rossi B, Angiari S, Dusi S, et al.: **Neutrophils promote Alzheimer's disease-like pathology and cognitive decline via LFA-1 integrin**. *Nat Med* 2015, **21**:880–886.

56. Rossi B, Angiari S, Zenaro E, Budui SL, Constantin G: **Vascular inflammation in central nervous system diseases: adhesion receptors controlling leukocyte-endothelial interactions**. *J Leukoc Biol* 2011, **89**:539–556.

57. Fabene PF, Mora GN, Martinello M, Rossi B, Merigo F, Ottoboni L, Bach S, Angiari S, Benati D, Chakir A, et al.: **A role for leukocyte-endothelial adhesion mechanisms in epilepsy**. *Nat Med* 2008, **14**:1377–1383.

58. Talukdar S, Oh DY, Bandyopadhyay G, Li D, Xu J, McNelis J, Lu M, Li P, Yan Q, Zhu Y, et al.: **Neutrophils mediate insulin resistance in mice fed a high-fat diet through secreted elastase**. *Nat Med* 2012, **18**:1407–1412.

59. Kordonowy LL, Burg E, Lenox CC, Gauthier LM, Petty JM, Antkowiak M, Palvinskaya T, Ubags N, Rincón M, Dixon AE, et al.: **Obesity is associated with neutrophil dysfunction and attenuation of murine acute lung injury**. *Am J Respir Cell Mol Biol* 2012, **47**:120–127.

60. Christoffersson G, Vågesjö E, Vandooren J, Lidén M, Massena S, Reinert RB, Brissova M, Powers AC, Opdenakker G, Phillipson M: **VEGF-Arecruits a proangiogenic MMP-9-delivering neutrophil subset that induces angiogenesis in transplanted hypoxic tissue**.

Blood 2012, **120**:4653–4662.

61. Fournier BM, Parkos CA: **The role of neutrophils during intestinal inflammation**. *Mucosal Immunol* 2012, **5**:354–366.

62. Jamieson T, Clarke M, Steele CW, Samuel MS, Neumann J, Jung A, Huels D, Olson MF, Das S, Nibbs RJB, et al.: **Inhibition of CXCR2 profoundly suppresses inflammation-driven and spontaneous tumorigenesis**. *J Clin Invest* 2012, **122**:3127–3144.

63. Katoh H, Wang D, Daikoku T, Sun H, Dey SK, DuBois RN: **CXCR2-Expressing Myeloid-Derived Suppressor Cells Are Essential to Promote Colitis-Associated Tumorigenesis**. *Cancer Cell* 2013, **24**:631–644.

64. Luppi F, Longo AM, de Boer WI, Rabe KF, Hiemstra PS: **Interleukin-8 stimulates cell proliferation in non-small cell lung cancer through epidermal growth factor receptor transactivation**. *Lung Cancer* 2007, **56**:25–33.

65. Knaapen AM, Güngör N, Schins RPF, Borm PJA, Van Schooten FJ: **Neutrophils and respiratory tract DNA damage and mutagenesis: A review**. *Mutagenesis* 2006, **21**:225–236.

66. Mangerich A, Knutson CG, Parry NM, Muthupalani S, Ye W, Prestwich E, Cui L, McFaline JL, Mobley M, Ge Z, et al.: **Infection-induced colitis in mice causes dynamic and tissue-specific changes in stress response and DNA damage leading to colon cancer**. *Proc Natl Acad Sci U S A* 2012, **109**.

67. Deryugina EI, Zajac E, Juncker-Jensen A, Kupriyanova TA, Welter L, Quigley JP: **Tissue-Infiltrating Neutrophils Constitute the Major In Vivo Source of Angiogenesis-Inducing MMP-9 in the Tumor Microenvironment**. *Neoplasia* 2014, **16**:771–788.

68. Houghton AMG, Rzymkiewicz DM, Ji H, Gregory AD, Egea EE, Metz HE, Stolz DB, Land SR, Marconcini LA, Kliment CR, et al.: **Neutrophil elastase-mediated degradation of IRS-1 accelerates lung tumor growth**. *Nat Med* 2010, **16**:219–223.

69. Lichtenstein A, Seelig M, Berek J, Zighelboim J: **Human neutrophil-mediated lysis of ovarian cancer cells**. *Blood* 1989, **74**:805–809.

70. Suttmann H, Riemensberger J, Bentien G, Schmaltz D, Stöckle M, Jocham D, Böhle A, Brandau S: **Neutrophil granulocytes are required for effective Bacillus Calmette-Guérin immunotherapy of bladder cancer and orchestrate local immune responses**. *Cancer Res* 2006, **66**:8250–8257.

71. Fridlender ZG, Sun J, Kim S, Kapoor V, Cheng G, Ling L, Worthen GS, Albelda SM: **Polarization of Tumor-Associated Neutrophil Phenotype by TGF-β: "N1" versus "N2" TAN**. *Cancer Cell* 2009, **16**:183–194.

72. Eruslanov EB, Bhojnagarwala PS, Quatromoni JG, Stephen TL, Ranganathan A, Deshpande C, Akimova T, Vachani A, Litzky L, Hancock WW, et al.: **Tumor-associated neutrophils stimulate T cell responses in early-stage human lung cancer**. *J Clin Invest* 2014, **124**:5466–5480.

73. Yan B, Wei J-J, Yuan Y, Sun R, Li D, Luo J, Liao S-J, Zhou Y-H, Shu Y, Wang Q, et al.: **IL-6 Cooperates with G-CSF To Induce Protumor Function of Neutrophils in Bone Marrow by Enhancing STAT3 Activation**. *J Immunol* 2013, **190**:5882–5893.

74. Nozawa H, Chiu C, Hanahan D: **Infiltrating neutrophils mediate the initial angiogenic switch in a mouse model of multistage carcinogenesis**. *Proc Natl Acad Sci U S A* 2006, **103**:12493–12498.

75. Coffelt SB, Kersten K, Doornebal CW, Weiden J, Vrijland K, Hau CS, Verstegen NJM, Ciampricotti M, Hawinkels LJAC, Jonkers J, et al.: **IL-17-producing γδ T cells and neutrophils conspire to promote breast cancer metastasis**. *Nature* 2015, **522**:345–348.

76. Spicer JD, McDonald B, Cools-Lartigue JJ, Chow SC, Giannias B, Kubes P, Ferri LE: **Neutrophils promote liver metastasis via Mac-1-mediated interactions with circulating tumor cells**. *Cancer Res* 2012, **72**:3919–3927.

77. Spiegel A, Brooks MW, Houshyar S, Reinhardt F, Ardolino M, Fessler E, Chen MB, Krall JA, Decock J, Zervantonakis IK, et al.: **Neutrophils suppress intraluminal NK cell-mediated tumor cell clearance and enhance extravasation of disseminated carcinoma cells**. *Cancer Discov* 2016, **6**:630–649.

78. Cools-Lartigue J, Spicer J, McDonald B, Gowing S, Chow S, Giannias B, Bourdeau F, Kubes P, Ferri L: **Neutrophil extracellular traps sequester circulating tumor cells and promote metastasis**. *J Clin Invest* 2013, **123**:3446–3458.

79. Nicolás-Ávila JÁ, Adrover JM, Hidalgo A: **Neutrophils in Homeostasis, Immunity, and Cancer**. *Immunity* 2017, **46**:15–28.

80. DiStasi MR, Ley K: **Opening the flood-gates: how neutrophil-endothelial interactions regulate permeability**. *Trends Immunol* 2009, **30**:547–556.

81. Alon R, Ley K: **Cells on the run: shear-regulated integrin activation in leukocyte rolling and arrest on endothelial cells**. *Curr Opin Cell Biol* 2008, **20**:525–532.

82. Abram CL, Lowell CA: **The Ins and Outs of Leukocyte Integrin Signaling**. *Annu Rev Immunol* 2009, **27**:339–362.

83. Zarbock A, Ley K: **Neutrophil adhesion and activation under flow.** *Microcirculation* 2009, **16**:31–42.

84. Lacy P, Eitzen G: **Control of granule exocytosis in neutrophils**. *Front Biosci* 2008, **13**:5559–5570.

85. Mehta D, Malik AB: **Signaling mechanisms regulating endothelial permeability**. *Physiol Rev* 2006, **86**:279–367.

86. Vandenbroucke E, Mehta D, Minshall R, Malik AB: **Regulation of endothelial junctional permeability**. In *Annals of the New York Academy of Sciences*. 2008:134–145.

87. Herman B, Bibert S, Concord E, Dublet B, Weidenhaupt M, Vernet T, Gulino-Debrac D: **Identification of proteases involved in the proteolysis of vascular endothelium cadherin during neutrophil transmigration**. *J Biol Chem* 2003, **278**:14002–14012.

88. Kaynar AM, Houghton AMG, Lum EH, Pitt BR, Shapiro SD: **Neutrophil elastase is needed for neutrophil emigration into lungs in ventilator-induced lung injury**. *Am J Respir Cell Mol Biol* 2008, **39**:53–60.

89. Gautam N, Herwald H, Hedqvist P, Lindbom L: **Signaling via β2 integrins triggers neutrophil-dependent alteration in endothelial barrier function**. *J Exp Med* 2000,

191:1829–1839.

90. Gautam N, Maria Olofsson A, Herwald H, Iversen LF, Lundgren-Åkerlund E, Hedqvist P, Arfors KE, Flodgaard H, Lindbom L: **Heparin-binding protein (HBP/CAP37): A missing link in neutrophil-evoked alteration of vascular permeability**. *Nat Med* 2001, **7**:1123–1127.

91. Hirahashi J, Mekala D, Van Ziffle J, Xiao L, Saffaripour S, Wagner DD, Shapiro SD, Lowell C, Mayadas TN: **Mac-1 Signaling via Src-Family and Syk Kinases Results in Elastase-Dependent Thrombohemorrhagic Vasculopathy**. *Immunity* 2006, **25**:271–283.

92. Nagase T, Uozumi N, Ishii S, Kume K, Izumi T, Ouchi Y, Shimizu T: **Acute lung injury by sepsis and acid aspiration: A key role for cytosolic phospholipase A2**. *Nat Immunol* 2000, **1**:42–45.

93. Benjamim CF, Canetti C, Cunha FQ, Kunkel SL, Peters-Golden M: **Opposing and Hierarchical Roles of Leukotrienes in Local Innate Immune versus Vascular Responses in a Model of Sepsis**. *J Immunol* 2005, **174**:1616–1620.

94. Zarbock A, Singbartl K, Ley K: **Complete reversal of acid-induced acute lung injury by blocking of platelet-neutrophil aggregation**. *J Clin Invest* 2006, **116**:3211–3219.

95. Funk CD: **Prostaglandins and leukotrienes: Advances in eicosanoid biology**. *Science* 2001, **294**:1871–1875.

96. Wedmore C V., Williams TJ: **Control of vascular permeability by polymorphonuclear leukocytes in inflammation**. *Nature* 1981, **289**:646–650.

97. Björk J, Hedqvist P, Arfors KE: **Increase in vascular permeability induced by leukotriene b4 and the role of polymorphonuclear leukocytes**. *Inflammation* 1982, **6**:189–200.

98. Rosengren S, Olofsson AM, Von Andrian UH, Lundgren-Akerlund E, Arfors KE: **Leukotriene B4-induced neutrophil-mediated endothelial leakage in vitro and in vivo**. *J Appl Physiol* 1991, **71**:1322–1330.

99. Tapper H, Karlsson A, Mörgelin M, Flodgaard H, Herwald H: **Secretion of heparin-binding protein from human neutrophils is determined by its localization in**

azurophilic granules and secretory vesicles. *Blood* 2002, **99**:1785–1793.

100. Di Gennaro A, Kenne E, Wan M, Soehnlein O, Lindbom L, Haeggström JZ: **Leukotriene B 4 -induced changes in vascular permeability are mediated by neutrophil release of heparin-binding protein (HBP/CAP37/azurocidin)** . *FASEB J* 2009, **23**:1750–1757.

101. Olofsson AM, Vestberg M, Herwald H, Rygaard J, David G, Arfors KE, Linde V, Flodgaard H, Dedio J, Müller-Esterl W, et al.: **Heparin-binding protein targeted to mitochondrial compartments protects endothelial cells from apoptosis**. *J Clin Invest* 1999, **104**:885–894.

102. Hui Y, Cheng Y, Smalera I, Jian W, Goldhahn L, FitzGerald GA, Funk CD: **Directed vascular expression of human cysteinyl leukotriene 2 receptor modulates endothelial permeability and systemic blood pressure**. *Circulation* 2004, **110**:3360–3366.

103. Moos MPW, Mewburn JD, Kan FWK, Ishii S, Abe M, Sakimura K, Noguchi K, Shimizu T, Funk CD: **Cysteinyl leukotriene 2 receptor-mediated vascular permeability via transendothelial vesicle transport** . *FASEB J* 2008, **22**:4352–4362.

104. McColl SR, St-Onge M, Dussault A, Laflamme C, Bouchard L, Boulanger J, Pouliot M: **Immunomodulatory impact of the A 2A adenosine receptor on the profile of chemokines produced by neutrophils** . *FASEB J* 2006, **20**:187–189.

105. Scapini P, Lapinet-Vera JA, Gasperini S, Calzetti F, Bazzoni F, Cassatella MA: **The neutrophil as a cellular source of chemokines**. *Immunol Rev* 2000, **177**:195–203.

106. Koss M, Pfeiffer GR, Wang Y, Thomas ST, Yerukhimovich M, Gaarde WA, Doerschuk CM, Wang Q: **Ezrin/Radixin/Moesin Proteins Are Phosphorylated by TNF-α and Modulate Permeability Increases in Human Pulmonary Microvascular Endothelial Cells**. *J Immunol* 2006, **176**:1218–1227.

107. Petrache I, Birukova A, Ramirez SI, Garcia JGN, Verin AD: **The role of the microtubules in tumor necrosis factor-α-induced endothelial cell permeability**. *Am J Respir Cell Mol Biol* 2003, **28**:574–581.

108. Strieter RM, Polverini PJ, Kunkel SL, Arenberg DA, Burdick MD, Kasper J, Dzuiba J, Van

Damme J, Walz A, Marriott D, et al.: **The functional role of the ELR motif in CXC chemokine-mediated angiogenesis**. *J Biol Chem* 1995, **270**:27348–27357.

109. Belperio JA, Keane MP, Burdick MD, Londhe V, Xue YY, Li K, Phillips RJ, Strieter RM: **Critical role for CXCR2 and CXCR2 ligands during the pathogenesis of ventilator-induced lung injury**. *J Clin Invest* 2002, **110**:1703–1716.

110. Gavard J, Hou X, Qu Y, Masedunskas A, Martin D, Weigert R, Li X, Gutkind JS: **A Role for a CXCR2/Phosphatidylinositol 3-Kinase γ Signaling Axis in Acute and Chronic Vascular Permeability**. *Mol Cell Biol* 2009, **29**:2469–2480.

111. Schraufstatter IU, Trieu K, Sikora L, Sriramarao P, DiScipio R: **Complement C3a and C5a Induce Different Signal Transduction Cascades in Endothelial Cells**. *J Immunol* 2002, **169**:2102–2110.

112. MONSINJON T, GASQUE P, CHAN P, ISCHENKO A, BRADY JJ, FONTAINE M: **Regulation by complement C3a and C5a anaphylatoxins of cytokine production in human umbilical vein endothelial cells**. *FASEB J* 2003, **17**:1003–1014.

113. Oksjoki R, Laine P, Helske S, Vehmaan-Kreula P, Mäyränpää MI, Gasque P, Kovanen PT, Pentikäinen MO: **Receptors for the anaphylatoxins C3a and C5a are expressed in human atherosclerotic coronary plaques**. *Atherosclerosis* 2007, **195**:90–99.

114. Jain RK: **Molecular regulation of vessel maturation**. *Nat Med* 2003, **9**:685–693.

115. Bhandari V, Choo-Wing R, Lee CG, Zhu Z, Nedrelow JH, Chupp GL, Zhang X, Matthay MA, Ware LB, Homer RJ, et al.: **Hyperoxia causes angiopoietin 2-mediated acute lung injury and necrotic cell death**. *Nat Med* 2006, **12**:1286–1293.

116. Lemieux C, Maliba R, Favier J, Théorêt JF, Merhi Y, Sirois MG: **Angiopoietins can directly activate endothelial cells and neutrophils to promote proinflammatory responses**. *Blood* 2005, **105**:1523–1530.

117. Wang Q, Doerschuk CM: **The signaling pathways induced by neutrophil-endothelial cell adhesion**. *Antioxidants Redox Signal* 2002, **4**:39–47.

118. Matheny HE, Deem TL, Cook-Mills JM: **Lymphocyte Migration Through Monolayers of**

Endothelial Cell Lines Involves VCAM-1 Signaling Via Endothelial Cell NADPH Oxidase. *J Immunol* 2000, **164**:6550–6559.

119. Tudor KSRS, Hess KL, Cook-Mills JM: **Cytokines modulate endothelial cell intracellular signal transduction required for VCAM-1-dependent lymphocyte transendothelial migration**. *Cytokine* 2001, **15**:196–211.

120. Davenpeck KL, Sterbinsky SA, Bochner BS: **Rat neutrophils express α4 and β1 integrins and bind to vascular cell adhesion molecule-1 (VCAM-1) and mucosal addressin cell adhesion molecule-1 (MAdCAM-1)**. *Blood* 1998, **91**:2341–2346.

121. Nottebaum AF, Cagna G, Winderlich M, Gamp AC, Linnepe R, Polaschegg C, Filippova K, Lyck R, Engelhardt B, Kamenyeva O, et al.: **VE-PTP maintains the endothelial barrier via plakoglobin and becomes dissociated from VE-cadherin by leukocytes and by VEGF**. *J Exp Med* 2008, **205**:2929–2945.

122. Alcaide P, Auerbach S, Luscinskas FW: **Neutrophil recruitment under shear flow: it's all about endothelial cell rings and gaps.** *Microcirculation* 2009, **16**:43–57.

123. Hu G, Vogel SM, Schwartz DE, Malik AB, Minshall RD: **Intercellular adhesion molecule-1-dependent neutrophil adhesion to endothelial cells induces caveolae-mediated pulmonary vascular hyperpermeability**. *Circ Res* 2008, **102**.

124. Lindbom L: **Regulation of Vascular Permeability by Neutrophils in Acute Inflammation**. *Chem Immunol Allergy* 2003, **83**:146–166.

125. Gautam N, Hedqvist P, Lindbom L: **Kinetics of leukocyte-induced changes in endothelial barrier function**. *Br J Pharmacol* 1998, **125**:1109–1114.

126. Yang L, Froio RM, Sciuto TE, Dvorak AM, Alon R, Luscinskas FW: **ICAM-1 regulates neutrophil adhesion and transcellular migration of TNF-α-activated vascular endothelium under flow**. *Blood* 2005, **106**:584–592.

127. Shaw SK, Ma S, Kim MB, Rao RM, Hartman CU, Froio RM, Yang L, Jones T, Liu Y, Nusrat A, et al.: **Coordinated redistribution of leukocyte LFA-1 and endothelial cell ICAM-1 accompany neutrophil transmigration**. *J Exp Med* 2004, **200**:1571–1580.

128. Boueiz A, Hassoun PM: **Regulation of endothelial barrier function by reactive oxygen and nitrogen species**. *Microvasc Res* 2009, **77**:26–34.

129. Allingham MJ, van Buul JD, Burridge K: **ICAM-1-Mediated, Src- and Pyk2-Dependent Vascular Endothelial Cadherin Tyrosine Phosphorylation Is Required for Leukocyte Transendothelial Migration**. *J Immunol* 2007, **179**:4053–4064.

130. van Wetering S, van Buul JD, Quik S, Mul FPJ, Anthony EC, ten Klooster JP, Collard JG, Hordijk PL: **Reactive oxygen species mediate Rac-induced loss of cell-cell adhesion in primary human endothelial cells**. *J Cell Sci* 2002, **115**:1837–1846.

131. Kása A, Csortos C, Verin AD: **Cytoskeletal mechanisms regulating vascular endothelial barrier function in response to acute lung injury**. *Tissue Barriers* 2015, **3**.

132. Rao R: **Oxidative stress-induced disruption of epithelial and endothelial tight junctions**. *Front Biosci* 2008, **13**:7210–7226.

133. Boueiz A, Damarla M, Hassoun PM: **Xanthine oxidoreductase in respiratory and cardiovascular disorders**. *Am J Physiol - Lung Cell Mol Physiol* 2008, **294**.

134. Morris PE, Papadakos P, Russell JA, Wunderink R, Schuster DP, Truwit JD, Vincent JL, Bernard GR: **A double-blind placebo-controlled study to evaluate the safety and efficacy of L-2-oxothiazolidine-4-carboxylic acid in the treatment of patients with acute respiratory distress syndrome**. *Crit Care Med* 2008, **36**:782–788.

135. Conti V, Izzo V, Corbi G, Russomanno G, Manzo V, De Lise F, Di Donato A, Filippelli A: **Antioxidant supplementation in the treatment of aging-associated diseases**. *Front Pharmacol* 2016, **7**.

136. Wang L, Dudek SM: **Regulation of vascular permeability by sphingosine 1-phosphate**. *Microvasc Res* 2009, **77**:39–45.

137. Sanchez T, Skoura A, Wu MT, Casserly B, Harrington EO, Hla T: **Induction of vascular permeability by the sphingosine-1-phosphate receptor-2 (S1P2R) and its downstream effectors ROCK and PTEN**. *Arterioscler Thromb Vasc Biol* 2007, **27**:1312–1318.

138. Ohta A, Sitkovsky M: **Role of G-protein-coupled adenosine receptors in downregulation**

of inflammation and protection from tissue damage. *Nature* 2001, **414**:916–920.

139. Eltzschig HK, Eckle T, Mager A, Küper N, Karcher C, Weissmüller T, Boengler K, Schulz R, Robson SC, Colgan SP: **ATP release from activated neutrophils occurs via connexin 43 and modulates adenosine-dependent endothelial cell function**. *Circ Res* 2006, **99**:1100–1108.

140. Eltzschig HK, Ibla JC, Furuta GT, Leonard MO, Jacobson KA, Enjyoji K, Robson SC, Colgan SR: **Coordinated adenine nucleotide phosphohydrolysis and nucleoside signaling in posthypoxic endothelium: Role of ectonucleotidases and adenosine A 2B receptors**. *J Exp Med* 2003, **198**:783–796.

141. Su X, Johansen M, Looney MR, Brown EJ, Matthay MA: **CD47 Deficiency Protects Mice from Lipopolysaccharide-Induced Acute Lung Injury and Escherichia coli Pneumonia** . *J Immunol* 2008, **180**:6947–6953.

142. Phaniendra A, Jestadi DB, Periyasamy L: **Free Radicals: Properties, Sources, Targets, and Their Implication in Various Diseases**. *Indian J Clin Biochem* 2015, **30**:11–26.

143. Pham-Huy LA, He H, Pham-Huy C: **Free radicals, antioxidants in disease and health**. *Int J Biomed Sci* 2008, **4**:89–96.

144. Valko M, Leibfritz D, Moncol J, Cronin MTD, Mazur M, Telser J: **Free radicals and antioxidants in normal physiological functions and human disease**. *Int J Biochem Cell Biol* 2007, **39**:44–84.

145. Nordberg J, Arnér ESJ: **Reactive oxygen species, antioxidants, and the mammalian thioredoxin system**. *Free Radic Biol Med* 2001, **31**:1287–1312.

146. Finkel T, Holbrook NJ: **Oxidants, oxidative stress and the biology of ageing**. *Nature* 2000, **408**:239–247.

147. Bedard K, Krause KH: **The NOX family of ROS-generating NADPH oxidases: Physiology and pathophysiology**. *Physiol Rev* 2007, **87**:245–313.

148. Schrader M, Fahimi HD: **Peroxisomes and oxidative stress**. *Biochim Biophys Acta - Mol Cell Res* 2006, **1763**:1755–1766.

149. Cheeseman KH, Slater TF: **An introduction to free radical biochemistry**. *Br Med Bull* 1993, **49**:481–493.

150. Land ET: **Free radicals in biology and medicine**. *Int J Radiat Biol* 1990, **58**:725–725.

151. Hofer T, Badouard C, Bajak E, Ravanat JL, Mattsson Å, Cotgreave IA: **Hydrogen peroxide causes greater oxidation in cellular RNA than in DNA**. *Biol Chem* 2005, **386**:333–337.

152. Siems WG, Grune T, Esterbauer H: **4-Hydroxynonenal formation during ischemia and reperfusion of rat small intestine**. *Life Sci* 1995, **57**:785–789.

153. Butterfield DA, Koppal T, Howard B, Subramaniam R, Hall N, Hensley K, Yatin S, Allen K, Aksenov M, Aksenova M, et al.: **Structural and functional changes in proteins induced by free radical-mediated oxidative stress and protective action of the antioxidants N-tert-butyl-α-phenylnitrone and vitamin E**. In *Annals of the New York Academy of Sciences*. 1998:448–462.

154. Brodie AE, Reed DJ: **Cellular recovery of glyceraldehyde-3-phosphate dehydrogenase activity and thiol status after exposure to hydroperoxides**. *Arch Biochem Biophys* 1990, **276**:212–218.

155. Pryor WA, Jin X, Squadrito GL: **One- and two-electron oxidations of methionine by peroxynitrite**. *Proc Natl Acad Sci U S A* 1994, **91**:11173–11177.

156. Sims EA, Catalano PM: **Response to the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus.** *Diabetes Care* 1998, **21**:467–468.

157. Ahmed R: **The physiological and biochemical effects of diabetes on the balance between oxidative stress and antioxidant defense system**. *Med J Islam World Acad Sci* 2005, **15**:31–42.

158. Santiago-López D, Bautista-Martínez JA, Reyes-Hernandez CI, Aguilar-Martínez M, Rivas-Arancibia S: **Oxidative stress, progressive damage in the substantia nigra and plasma dopamine oxidation, in rats chronically exposed to ozone**. *Toxicol Lett* 2010, **197**:193–200.

159. Butterfield DA, Perluigi M, Sultana R: **Oxidative stress in Alzheimer's disease brain:**

New insights from redox proteomics. *Eur J Pharmacol* 2006, **545**:39–50.

160. Gonsette RE: **Neurodegeneration in multiple sclerosis: The role of oxidative stress and excitotoxicity**. *J Neurol Sci* 2008, **274**:48–53.

161. Papaharalambus CA, Griendling KK: **Basic Mechanisms of Oxidative Stress and Reactive Oxygen Species in Cardiovascular Injury**. *Trends Cardiovasc Med* 2007, **17**:48–54.

162. Ding Z, Liu S, Wang X, Khaidakov M, Dai Y, Mehta JL: **Oxidant stress in mitochondrial DNA damage, autophagy and inflammation in atherosclerosis**. *Sci Rep* 2013, **3**.

163. Mercer JR, Cheng KK, Figg N, Gorenne I, Mahmoudi M, Griffin J, Vidal-Puig A, Logan A, Murphy MP, Bennett M: **DNA damage links mitochondrial dysfunction to atherosclerosis and the metabolic syndrome**. *Circ Res* 2010, **107**:1021–1031.

164. Dzau VJ: **Tissue angiotensin and pathobiology of vascular disease a unifying hypothesis**. *Hypertension* 2001, **37**:1047–1052.

165. Touyz RM: **Reactive oxygen species, vascular oxidative stress, and redox signaling in hypertension: What is the clinical significance?** *Hypertension* 2004, **44**:248–252.

166. Stamp LK, Khalilova I, Tarr JM, Senthilmohan R, Turner R, Haigh RC, Winyard PG, Kettle AJ: **Myeloperoxidase and oxidative stress in rheumatoid arthritis**. *Rheumatol (United Kingdom)* 2012, **51**:1796–1803.

167. Hitchon CA, El-Gabalawy HS: **Oxidation in rheumatoid arthritis**. *Arthritis Res Ther* 2004, **6**:265–278.

168. Gupta SK, Trivedi D, Srivastava S, Joshi S, Halder N, Verma SD: **Lycopene attenuates oxidative stress induced experimental cataract development: An in vitro and in vivo study**. *Nutrition* 2003, **19**:794–799.

169. Rahman I, Biswas SK, Kode A: **Oxidant and antioxidant balance in the airways and airway diseases**. *Eur J Pharmacol* 2006, **533**:222–239.

170. Terada LS: **Specificity in reactive oxidant signaling: Think globally, act locally**. *J Cell Biol* 2006, **174**:615–623.

171. Hoagland IV LFM, Campa MJ, Gottlin EB, Herndon JE, Patz EF: **Haptoglobin and posttranslational glycan-modified derivatives as serum biomarkers for the diagnosis of nonsmall cell lung cancer**. *Cancer* 2007, **110**:2260–2268.

172. Brown NS, Jones A, Fujiyama C, Harris AL, Bicknell R: **Thymidine phosphorylase induces carcinoma cell oxidative stress and promotes secretion of angiogenic factors**. *Cancer Res* 2000, **60**:6298–6302.

173. Weyemi U, E. Redon C, R. Parekh P, Dupuy C, M. Bonner W: **NADPH Oxidases NOXs and DUOXs as Putative Targets for Cancer Therapy**. *Anticancer Agents Med Chem* 2013, **13**:502–514.

174. Eiján AM, Piccardo I, Doris Riveros M, Omar Sandes E, Porcella H, Adela Jasnis M, De Lustig ES, Malagrino H, Pasik L, Ricardo Casabé A: **Nitric oxide in patients with transitional bladder cancer**. *J Surg Oncol* 2002, **81**:203–208.

175. Sadik CD, Kim ND, Luster AD: **Neutrophils cascading their way to inflammation**. *Trends Immunol* 2011, **32**:452–460.

176. Sanz MJ, Kubes P: **Neutrophil-active chemokines in in vivo imaging of neutrophil trafficking**. *Eur J Immunol* 2012, **42**:278–283.

177. Rajarathnam K, Sepuru KM, Joseph PRB, Sawant K V., Brown AJ: **Glycosaminoglycan Interactions Fine-Tune Chemokine-Mediated Neutrophil Trafficking: Structural Insights and Molecular Mechanisms**. *J Histochem Cytochem* 2018, **66**:229–239.

178. Wilson GC, Kuboki S, Freeman CM, Nojima H, Schuster RM, Edwards MJ, Lentsch AB: **CXC Chemokines Function as a Rheostat for Hepatocyte Proliferation and Liver Regeneration**. *PLoS One* 2015, **10**.

179. Jaeschke H: **Inflammation in response to hepatocellular apoptosis**. *Hepatology* 2002, **35**:964–966.

180. O'Brien KM, Allen KM, Rockwell CE, Towery K, Luyendyk JP, Copple BL: **IL-17A synergistically enhances bile acid-induced inflammation during obstructive cholestasis**. *Am J Pathol* 2013, **183**:1498–1507.

181. Yoshidome H, Kato A, Edwards MJ, Lentsch AB: **Interleukin-10 suppresses hepatic ischemia/reperfusion injury in mice: Implications of a central role for nuclear factor κB**. *Hepatology* 1999, **30**:203–208.

182. Colletti LM, Cortis A, Lukacs N, Kunkel SL, Green M, Strieter RM: **Tumor necrosis factor up-regulates intercellular adhesion molecule 1, which is important in the neutrophil-dependent lung and liver injury associated with hepatic ischemia and reperfusion in the rat**. *Shock* 1998, **10**:182–191.

183. Ohno Y, Lee J, Fusunyan RD, MacDermott RP, Sanderson IR: **Macrophage inflammatory protein-2: Chromosomal regulation in rat small intestinal epithelial cells**. *Proc Natl Acad Sci U S A* 1997, **94**:10279–10284.

184. Lentsch AB, Yoshidome H, Cheadle WG, Miller FN, Edwards MJ: **Chemokine involvement in hepatic ischemia/reperfusion injury in mice: Roles for macrophage inflammatory protein-2 and Kupffer cells**. *Hepatology* 1998, **27**:507–512.

185. Milatovic S, Nanney LB, Yu Y, White JR, Richmond A: **Impaired healing of nitrogen mustard wounds in CXCR2 null mice**. *Wound Repair Regen* 2003, **11**:213–219.

186. Devalaraja RM, Nanney LB, Qian Q, Du J, Yu Y, Devalaraja MN, Richmond A: **Delayed wound healing in CXCR 2 knockout mice**. *J Invest Dermatol* 2000, **115**:234–244.

187. Frevert CW, Huang S, Danaee H, Paulauskis JD, Kobzik L: **Functional characterization of the rat chemokine KC and its importance in neutrophil recruitment in a rat model of pulmonary inflammation**. *J Immunol* 1995, **154**:335–344.

188. Zhao Z, Hyun JS, Satsu H, Kakuta S, Shimizu M: **Oral exposure to cadmium chloride triggers an acute inflammatory response in the intestines of mice, initiated by the over-expression of tissue macrophage inflammatory protein-2 mRNA**. *Toxicol Lett* 2006, **164**:144–154.

189. Ramos CDL, Fernandes KSS, Canetti C, Teixeira MM, Silva JS, Cunha F de Q: **Neutrophil recruitment in immunized mice depends on MiP-2 inducing the sequential release of MIP-1α, TNF-α and LTB4**. *Eur J Immunol* 2006, **36**:2025–2034.

190. Feng L, Xia Y, Yoshimura T, Wilson CB: **Modulation of neutrophil influx in glomerulonephritis in the rat with anti-macrophage inflammatory protein-2 (MIP-2) antibody**. *J Clin Invest* 1995, **95**:1009–1017.

191. Cole N, Hume EBH, Khan S, Garthwaite L, Conibear TCR, Willcox MDP: **The role of CXC chemokine receptor 2 in Staphylococcus aureus keratitis**. *Exp Eye Res* 2014, **127**:184–189.

192. Hosoki K, Itazawa T, Boldogh I, Sur S: **Neutrophil recruitment by allergens contribute to allergic sensitization and allergic inflammation**. *Curr Opin Allergy Clin Immunol* 2016, **16**:45–50.

193. Acharyya S, Oskarsson T, Vanharanta S, Malladi S, Kim J, Morris PG, Manova-Todorova K, Leversha M, Hogg N, Seshan VE, et al.: **A CXCL1 paracrine network links cancer chemoresistance and metastasis**. *Cell* 2012, **150**:165–178.

194. Schiffmann E, Corcoran BA, Wahl SM: **N formylmethionyl peptides as chemoattractants for leucocytes**. *Proc Natl Acad Sci U S A* 1975, **72**:1059–1062.

195. Ye RD, Boulay F, Ji MW, Dahlgren C, Gerard C, Parmentier M, Serhan CN, Murphy PM: **International union of basic and clinical pharmacology. LXXIII. Nomenclature for the formyl peptide receptor (FPR) family**. *Pharmacol Rev* 2009, **61**:119–161.

196. Cattaneo F, Guerra G, Ammendola R: **Expression and signaling of formyl-peptide receptors in the brain**. *Neurochem Res* 2010, **35**:2018–2026.

197. Kim SD, Kim JM, Jo SH, Lee HY, Lee SY, Shim JW, Seo S-K, Yun J, Bae Y-S: **Functional Expression of Formyl Peptide Receptor Family in Human NK Cells**. *J Immunol* 2009, **183**:5511–5517.

198. Bradford PG, Rubin RP: **Characterization of formylmethionyl-leucyl-phenylalanine stimulation of inositol trisphosphate accumulation in rabbit neutrophils**. *Mol Pharmacol* 1985, **27**:74–78.

199. Showell HJ, Freer RJ, Zigmond SH, Schiffmann E, Aswanikumar S, Corcoran B, Becker EL: **The structure-activity relations of synthetic peptides as chemotactic factors and**

inducers of lysosomal enzyme secretion for neutrophils*. *J Exp Med* 1976, **143**:1154–1169.

200. Freer RJ, Day AR, Radding JA, Schiffmann E, Aswanikumar E, Showell HJ, Becker EL: **Further Studies on the Structural Requirements for Synthetic Peptide Chemoattractants**. *Biochemistry* 1980, **19**:2404–2410.
201. Clark RB, Knoll BJ: **Measurement of receptor desensitization and internalization in intact cells**. *Methods Enzymol* 2002, **343**:506–529.
202. Zigmond SH, Levitsky HI, Kreel BJ: **Cell polarity: An examination of its behavioral expression and its consequences for polymorphonuclear leukocyte chemotaxis**. *J Cell Biol* 1981, **89**:585–592.
203. Hsu MH, Chiang SC, Ye RD, Prossnitz ER: **Phosphorylation of the N-formyl peptide receptor is required for receptor internalization but not chemotaxis**. *J Biol Chem* 1997, **272**:29426–29429.
204. Neptune ER, Bourne HR: **Receptors induce chemotaxis by releasing the βγ subunit of Gi, not by activating Gq or Gs**. *Proc Natl Acad Sci U S A* 1997, **94**:14489–14494.
205. Boxer LA, Yoder M, Bonsib S, Schmidt M, Ho P, Jersild R, Baehner RL: **Effects of a chemotactic factor, N-formylmethionyl peptide, on adherence, superoxide anion generation, phagocytosis, and microtubule assembly of human polymorphonuclear leukocytes**. *J Lab Clin Med* 1979, **93**:506–514.
206. Lehmeyer JE, Snyderman R, Johnston RB: **Stimulation of neutrophil oxidative metabolism by chemotactic peptides: Influence of calcium ion concentration and cytochalasin B and comparison with stimulation by phorbol myristate acetate**. *Blood* 1979, **54**:35–45.
207. Karnad AB, Hartshorn KL, Wright J, Myers JB, Schwartz JH, Tauber AI: **Priming of human neutrophils with N-formyl-methionyl-leucyl-phenylalanine by a calcium-independent, pertussis toxin-insensitive pathway**. *Blood* 1989, **74**:2519–2526.
208. Borregaard N, Lollike K, Kjeldsen L, Sengeløv H, Bastholm L, Nielsen MH, Bainton DF:

Human neutrophil granules and secretory vesicles. *Eur J Haematol* 1993, **51**:187–198.

209. Lloyd AR, Oppenheim JJ: **Poly's lament: the neglected role of the polymorphonuclear neutrophil in the afferent limb of the immune response**. *Immunol Today* 1992, **13**:169–172.

210. Cassatella MA, Bazzoni F, Ceska M, Ferro I, Baggiolini M, Berton G: **IL-8 production by human polymorphonuclear leukocytes. The chemoattractant formyl-methionyl-leucyl-phenylalanine induces the gene expression and release of IL-8 through a pertussis toxin-sensitive pathway.** *J Immunol* 1992, **148**:3216–20.

211. Kettritz R, Falk RJ, Jennette JC, Gaido ML: **Neutrophil superoxide release is required for spontaneous and FMLP-mediated but not for TNFα-mediated apoptosis**. *J Am Soc Nephrol* 1997, **8**:1091–1100.

212. Okkenhaug K: **Signaling by the Phosphoinositide 3-Kinase Family in Immune Cells**. *Annu Rev Immunol* 2013, **31**:675–704.

213. Vanhaesebroeck B, Guillermet-Guibert J, Graupera M, Bilanges B: **The emerging mechanisms of isoform-specific PI3K signalling**. *Nat Rev Mol Cell Biol* 2010, **11**:329–341.

214. Lemmon MA: **Membrane recognition by phospholipid-binding domains**. *Nat Rev Mol Cell Biol* 2008, **9**:99–111.

215. Vadas O, Burke JE, Zhang X, Berndt A, Williams RL: **Structural biology structural basis for activation and inhibition of class I phosphoinositide 3-kinases**. *Sci Signal* 2011, **4**.

216. Krugmann S, Anderson KE, Ridley SH, Risso N, McGregor A, Coadwell J, Davidson K, Eguinoa A, Ellson CD, Lipp P, et al.: **Identification of ARAP3, a novel PI3K effector regulating both Arf and Rho GTPases, by selective capture on phosphoinositide affinity matrices**. *Mol Cell* 2002, **9**:95–108.

217. Allam A, Marshall AJ: **Role of the adaptor proteins Bam32, TAPP1 and TAPP2 in lymphocyte activation**. *Immunol Lett* 2005, **97**:7–17.

218. Hawkins PT, Stephens LR: **PI3K signalling in inflammation**. *Biochim Biophys Acta - Mol*

Cell Biol Lipids 2015, **1851**:882–897.

219. Stephens L, Milne L, Hawkins P: **Moving towards a Better Understanding of Chemotaxis**. *Curr Biol* 2008, **18**.

220. Hirsch E, Katanaev VL, Garlanda C, Azzolino O, Pirola L, Silengo L, Sozzani S, Mantovani A, Altruda F, Wymann MP: **Central role for G protein-coupled phosphoinositide 3-kinase γ in inflammation**. *Science* 2000, **287**:1049–1052.

221. Li Z, Jiang H, Xie W, Zhang Z, Smrcka A V., Wu D: **Roles of PLC-β2 and -β3 and PI3kγ in chemoattractant-mediated signal transduction**. *Science* 2000, **287**:1046–1049.

222. Sasaki T, Irie-Sasaki J, Jones RG, Oliveira-Dos-Santos AJ, Stanford WL, Bolon B, Wakeham A, Itie A, Bouchard D, Kozieradzki I, et al.: **Function of PI3Kγ in thymocyte development, T cell activation, and neutrophil migration**. *Science* 2000, **287**:1040–4046.

223. Ferguson GJ, Milne L, Kulkarni S, Sasaki T, Walker S, Andrews S, Crabbe T, Finan P, Jones G, Jackson S, et al.: **PI(3)Kγ has an important context-dependent role in neutrophil chemokinesis**. *Nat Cell Biol* 2007, **9**:86–91.

224. Smith DF, Deem TL, Bruce AC, Reutershan J, Wu D, Ley K: **Leukocyte phosphoinositide-3 kinase γ is required for chemokine-induced, sustained adhesion under flow in vivo**. *J Leukoc Biol* 2006, **80**:1491–1499.

225. Barberis L, Pasquali C, Bertschy-Meier D, Cuccurullo A, Costa C, Ambrogio C, Vilbois F, Chiarle R, Wymann M, Altruda F, et al.: **Leukocyte transmigration is modulated by chemokine-mediated PI3Kγ-dependent phosphorylation of vimentin**. *Eur J Immunol* 2009, **39**:1136–1146.

226. Sadhu C, Masinovsky B, Dick K, Sowell CG, Staunton DE: **Essential Role of Phosphoinositide 3-Kinase δ in Neutrophil Directional Movement**. *J Immunol* 2003, **170**:2647–2654.

227. Liu L, Puri KD, Penninger JM, Kubes P: **Leukocyte PI3Kγ and PI3Kδ have temporally distinct roles for leukocyte recruitment in vivo**. *Blood* 2007, **110**:1191–1198.

228. Puri KD, Doggett TA, Huang CY, Douangpanya J, Hayflick JS, Turner M, Penninger J,

Diacovo TG: **The role of endothelial PI3Kγ activity in neutrophil trafficking**. *Blood* 2005, **106**:150–157.

229. di Blasio L, Gagliardi PA, Puliafito A, Sessa R, Seano G, Bussolino F, Primo L: **PDK1 regulates focal adhesion disassembly by modulating endocytosis of αvβ3 integrin**. *J Cell Sci* 2015, **128**:863–877.
230. Widmann C, Gibson S, Jarpe MB, Johnson GL: **Mitogen-activated protein kinase: Conservation of a three-kinase module from yeast to human**. *Physiol Rev* 1999, **79**:143–180.
231. Cargnello M, Roux PP: **Activation and Function of the MAPKs and Their Substrates, the MAPK-Activated Protein Kinases**. *Microbiol Mol Biol Rev* 2011, **75**:50–83.
232. Robbins DJ, Zhen E, Owaki H, Vanderbilt CA, Ebert D, Geppert TD, Cobb MH: **Regulation and properties of extracellular signal-regulated protein kinases 1 and 2 in vitro**. *J Biol Chem* 1993, **268**:5097–5106.
233. Coulombe P, Meloche S: **Atypical mitogen-activated protein kinases: Structure, regulation and functions**. *Biochim Biophys Acta - Mol Cell Res* 2007, **1773**:1376–1387.
234. Gaestel M: **Specificity of signaling from MAPKs to MAPKAPKs: Kinases' Tango Nuevo**. *Front Biosci* 2008, **13**:6050–6059.
235. Roux PP, Blenis J: **ERK and p38 MAPK-Activated Protein Kinases: a Family of Protein Kinases with Diverse Biological Functions**. *Microbiol Mol Biol Rev* 2004, **68**:320–344.
236. Carriere A, Ray H, Blenis J, Roux PP: **The RSK factors of activating the Ras/MAPK signaling cascade**. *Front Biosci* 2008, **13**:4258–4275.
237. Arthur JSC: **MSK activation and physiological roles**. *Front Biosci* 2008, **13**:5866–5879.
238. Buxade M, Parra-Palau JL, Proud CG: **The Mnks: MAP kinase-interacting kinases (MAP kinase signal-integrating kinases)**. *Front Biosci* 2008, **13**:5359–5374.
239. Ronkina N, Kotlyarov A, Gaestel M: **MK2 and MK3 - A pair of isoenzymes?** *Front Biosci* 2008, **13**:5511–5521.
240. Perander M, Keyse SM, Seternes OM: **Does MK5 reconcile classical and atypical MAP**

kinases? *Front Biosci* 2008, **13**:4617–4624.

241. Chen RH, Sarnecki C, Blenis J: **Nuclear localization and regulation of erk- and rsk-encoded protein kinases.** *Mol Cell Biol* 1992, **12**:915–927.

242. Lenormand P, Sardet C, Pages G, L'Allemain G, Brunet A, Pouyssegur J: **Growth factors induce nuclear translocation of MAP kinases (p42(mapk) and p44(mapk)) but not their activator MAP kinase kinase (p45(mapkk)) in fibroblasts**. *J Cell Biol* 1993, **122**:1079–1088.

243. Choi C, Helfman DM: **The Ras-ERK pathway modulates cytoskeleton organization, cell motility and lung metastasis signature genes in MDA-MB-231 LM2**. *Oncogene* 2014, **33**:3668–3676.

244. Nakamae-Akahori M, Kato T, Masuda S, Sakamoto E, Kutsuna H, Hato F, Nishizawa Y, Hino M, Kitagawa S: **Enhanced neutrophil motility by granulocyte colony-stimulating factor: The role of extracellular signal-regulated kinase and phosphatidylinositol 3-kinase**. *Immunology* 2006, **119**:393–403.

245. Mizuno R, Kamioka Y, Kabashima K, Imajo M, Sumiyama K, Nakasho E, Ito T, Hamazaki Y, Okuchi Y, Sakai Y, et al.: **In vivo imaging reveals PKA regulation of ERK activity during neutrophil recruitment to inflamed intestines**. *J Exp Med* 2014, **211**:1123–1136.

246. Hii CS, Anson DS, Costabile M, Mukaro V, Dunning K, Ferrante A: **Characterization of the MEK5-ERK5 module in human neutrophils and its relationship to ERK1/ERK2 in the chemotactic response**. *J Biol Chem* 2004, **279**:49825–49834.

247. Arndt PG, Young SK, Worthen GS: **Regulation of Lipopolysaccharide-Induced Lung Inflammation by Plasminogen Activator Inhibitor-1 through a JNK-Mediated Pathway**. *J Immunol* 2005, **175**:4049–4059.

248. Lokuta MA: **TNF- promotes a stop signal that inhibits neutrophil polarization and migration via a p38 MAPK pathway**. *J Leukoc Biol* 2005, **78**:210–219.

249. Wang X, Qin W, Xu X, Xiong Y, Zhang Y, Zhang H, Sun B: **Endotoxin-induced autocrine ATP signaling inhibits neutrophil chemotaxis through enhancing myosin light chain**

phosphorylation. *Proc Natl Acad Sci U S A* 2017, **114**:4483–4488.

250. Xu N, Hossain M, Liu L: **Pharmacological inhibition of p38 mitogen-activated protein kinases affects KC/CXCL1-induced intraluminal crawling, transendothelial migration, and chemotaxis of neutrophils in vivo**. *Mediators Inflamm* 2013, **2013**.

251. Ward RA, Nakamura M, McLeish KR: **Priming of the neutrophil respiratory burst involves p38 mitogen-activated protein kinase-dependent exocytosis of flavocytochrome b558-containing granules**. *J Biol Chem* 2000, **275**:36713–36719.

252. Fumagalli L, Zhang H, Baruzzi A, Lowell CA, Berton G: **The Src Family Kinases Hck and Fgr Regulate Neutrophil Responses to N -Formyl-Methionyl-Leucyl-Phenylalanine** . *J Immunol* 2007, **178**:3874–3885.

253. Malek M, Kielkowska A, Chessa T, Anderson KE, Barneda D, Pir P, Nakanishi H, Eguchi S, Koizumi A, Sasaki J, et al.: **PTEN Regulates PI(3,4)P2 Signaling Downstream of Class I PI3K**. *Mol Cell* 2017, **68**:566-580.e10.

254. Berra E, Diaz-Meco MT, Moscat J: **The activation of p38 and apoptosis by the inhibition of Erk is antagonized by the phosphoinositide 3-kinase/Akt pathway**. *J Biol Chem* 1998, **273**:10792–10797.

255. Alvarado-Kristensson M, Porn-Ares MI, Grethe S, Smith D, Zheng L, Andersson T: **p38 Mitogen-activated protein kinase and phosphatidylinositol 3-kinase activities have opposite effects on human neutrophil apoptosis.** *FASEB J* 2002, **16**:129–131.

256. Zhang TT, Li H, Cheung SM, Costantini JL, Hou S, Al-Alwan M, Marshall AJ: **Phosphoinositide 3-kinase-regulated adapters in lymphocyte activation**. *Immunol Rev* 2009, **232**:255–272.

257. Marshall AJ, Niiro H, Lerner CG, Yun TJ, Thomas S, Disteche CM, Clark EA: **A novel B lymphocyte-associated adaptor protein, Bam32, regulates antigen receptor signaling downstream of phosphatidylinositol 3-kinase**. *J Exp Med* 2000, **191**:1319–1331.

258. Baraldi E, Carugo KD, Hyvönen M, Surdo P Lo, Riley AM, Potter BVL, O'Brien R, Ladbury JE, Saraste M: **Structure of the PH domain from Bruton's tyrosine kinase in**

complex with inositol 1,3,4,5-tetrakisphosphate. *Structure* 1999, **7**:449–460.

259. Ferguson KM, Kavran JM, Sankaran VG, Fournier E, Isakoff SJ, Skolnik EY, Lemmon MA: **Structural basis for discrimination of 3-phosphoinositides by pleckstrin homology domains**. *Mol Cell* 2000, **6**:373–384.

260. Fournier E, Isakoff SJ, Ko K, Cardinale CJ, Inghirami GG, Li Z, Curotto De Lafaille MA, Skolnik EY: **The B Cell SH2/PH Domain-Containing Adaptor Bam32/DAPP1 Is Required for T Cell-Independent II Antigen Responses**. *Curr Biol* 2003, **13**:1858–1866.

261. Hou S, Pauls SD, Liu P, Marshall AJ: **The PH domain adaptor protein Bam32/DAPP1 functions in mast cells to restrain Fce{open}RI-induced calcium flux and granule release**. *Mol Immunol* 2010, **48**:89–97.

262. Ortner D, Grabher D, Hermann M, Kremmer E, Hofer S, Heufler C: **The Adaptor Protein Bam32 in Human Dendritic Cells Participates in the Regulation of MHC Class I-Induced CD8 + T Cell Activation** . *J Immunol* 2011, **187**:3972–3978.

263. Han A, Saijo K, Mecklenbräuker I, Tarakhovsky A, Nussenzweig MC: **Bam32 links the B cell receptor to ERK and JNK and mediates B cell proliferation but not survival**. *Immunity* 2003, **19**:621–632.

264. Stephens LR, Anderson KE, Hawkins PT: **Src Family Kinases Mediate Receptor-stimulated, Phosphoinositide 3-Kinase-dependent, Tyrosine Phosphorylation of Dual Adaptor for Phosphotyrosine and 3-Phosphoinositides-1 in Endothelial and B Cell Lines**. *J Biol Chem* 2001, **276**:42767–42773.

265. Dowler S, Montalvo L, Cantrell D, Morrice N, Alessi DR: **Phosphoinositide 3-kinase-dependent phosphorylation of the dual adaptor for phosphotyrosine and 3-phosphoinositides by the Src family of tyrosine kinase**. *Biochem J* 2000, **349**:605–610.

266. Allam A, Niiro H, Clark EA, Marshall AJ: **The adaptor protein Bam32 regulates Rac1 activation and actin remodeling through a phosphorylation-dependent mechanism**. *J Biol Chem* 2004, **279**:39775–39782.

267. Arana E, Vehlow A, Harwood NE, Vigorito E, Henderson R, Turner M, Tybulewicz VLLJ,

Batista FD: **Activation of the Small GTPase Rac2 via the B Cell Receptor Regulates B Cell Adhesion and Immunological-Synapse Formation**. *Immunity* 2008, **28**:88–99.

268. Lin KBL, Freeman SA, Zabetian S, Brugger H, Weber M, Lei V, Dang-Lawson M, Tse KWK, Santamaria R, Batista FD, et al.: **The Rap GTPases Regulate B Cell Morphology, Immune-Synapse Formation, and Signaling by Particulate B Cell Receptor Ligands**. *Immunity* 2008, **28**:75–87.

269. Al-Alwan M, Hou S, Zhang T, Makondo K, Marshall AJ: **Bam32/DAPP1 Promotes B Cell Adhesion and Formation of Polarized Conjugates with T Cells**. *J Immunol* 2010, **184**:6961–6969.

270. Niiro H, Allam A, Stoddart A, Brodsky FM, Marshall AJ, Clark EA: **The B Lymphocyte Adaptor Molecule of 32 Kilodaltons (Bam32) Regulates B Cell Antigen Receptor Internalization**. *J Immunol* 2004, **173**:5601–5609.

271. Zhang T, Al-Alwan M, Marshall AJ: **The Pleckstrin Homology Domain Adaptor Protein Bam32/DAPP1 Is Required for Germinal Center Progression**. *J Immunol* 2010, **184**:164–172.

272. Sommers CL, Gurson JM, Surana R, Barda-Saad M, Lee J, Kishor A, Li W, Gasser AJ, Barr VA, Miyaji M, et al.: **Bam32: A novel mediator of Erk activation in T cells**. *Int Immunol* 2008, **20**:811–818.

273. Miyaji M, Kortum RL, Surana R, Li W, Woolard KD, Simpson RM, Samelson LE, Sommers CL: **Genetic evidence for the role of Erk activation in a lymphoproliferative disease of mice**. *Proc Natl Acad Sci U S A* 2009, **106**:14502–14507.

274. Gambardella L, Vermeren S: **Molecular players in neutrophil chemotaxis-focus on PI3K and small GTPases**. *J Leukoc Biol* 2013, **94**:603–612.

275. McCormick B, Chu JY, Vermeren S: **Cross-talk between Rho GTPases and PI3K in the neutrophil**. *Small GTPases* 2019, **10**:187–195.

276. Gardiner EM, Pestonjamasp KN, Bohl BP, Chamberlain C, Hahn KM, Bokoch GM: **Spatial and temporal analysis of Rac activation during live neutrophil chemotaxis**. *Curr Biol*

2002, **12**:2029–2034.

277. Sun CX, Downey GP, Zhu F, Koh ALY, Thang H, Glogauer M: **Rac1 is the small GTPase responsible for regulating the neutrophil chemotaxis compass**. *Blood* 2004, **104**:3758–3765.

278. Glogauer M, Marchal CC, Zhu F, Worku A, Clausen BE, Foerster I, Marks P, Downey GP, Dinauer M, Kwiatkowski DJ: **Rac1 Deletion in Mouse Neutrophils Has Selective Effects on Neutrophil Functions**. *J Immunol* 2003, **170**:5652–5657.

279. Pestonjamasp KN, Forster C, Sun C, Gardiner EM, Bohl B, Weiner O, Bokoch GM, Glogauer M: **Rac1 links leading edge and uropod events through Rho and myosin activation during chemotaxis**. *Blood* 2006, **108**:2814–2820.

280. Roberts AW, Chaekyun K, Zhen L, Lowe JB, Kapur R, Petryniak B, Spaetti A, Pollock JD, Borneo JB, Bradford GB, et al.: **Deficiency of the hematopoietic cell-specific Rho family GTPase Rac2 is characterized by abnormalities in neutrophil function and host defense**. *Immunity* 1999, **10**:183–196.

281. Lawson CD, Donald S, Anderson KE, Patton DT, Welch HCE: **P-Rex1 and Vav1 Cooperate in the Regulation of Formyl-Methionyl-Leucyl-Phenylalanine–Dependent Neutrophil Responses**. *J Immunol* 2011, **186**:1467–1476.

282. Welch HCE, Condliffe AM, Milne LJ, Ferguson GJ, Hill K, Webb LMC, Okkenhaug K, Coadwell WJ, Andrews SR, Thelen M, et al.: **P-Rex1 regulates neutrophil function**. *Curr Biol* 2005, **15**:1867–1873.

283. Dong X, Mo Z, Bokoch G, Guo C, Li Z, Wu D: **P-Rex1 is a primary Rac2 guanine nucleotide exchange factor in mouse neutrophils**. *Curr Biol* 2005, **15**:1874–1879.

284. Kunisaki Y, Nishikimi A, Tanaka Y, Takii R, Noda M, Inayoshi A, Watanabe KI, Sanematsu F, Sasazuki T, Sasaki T, et al.: **DOCK2 is a Rac activator that regulates motility and polarity during neutrophil chemotaxis**. *J Cell Biol* 2006, **174**:647–652.

285. Gérard A, Van Der Kammen RA, Janssen H, Ellenbroek SI, Collard JG: **The Rac activator Tiam1 controls efficient T-cell trafficking and route of transendothelial migration**.

Blood 2009, **113**:6138–6147.

286. Costa C, Germena G, Martin-Conte EL, Molineris I, Bosco E, Marengo S, Azzolino O, Altruda F, Ranieri VM, Hirsch E: **The RacGAP ArhGAP15 is a master negative regulator of neutrophil functions**. *Blood* 2011, **118**:1099–1108.

287. Csépányi-Kömi R, Wisniewski É, Bartos B, Lévai P, Németh T, Balázs B, Kurz ARM, Bierschenk S, Sperandio M, Ligeti E: **Rac GTPase Activating Protein ARHGAP25 Regulates Leukocyte Transendothelial Migration in Mice**. *J Immunol* 2016, **197**:2807–2815.

288. Martinez Gakidis MA, Cullere X, Olson T, Wilsbacher JL, Zhang B, Moores SL, Ley K, Swat W, Mayadas T, Brugge JS: **Vav GEFs are required for β2 integrin-dependent functions of neutrophils**. *J Cell Biol* 2004, **166**:273–282.

289. Boespflug ND, Kumar S, McAlees JW, Phelan JD, Grimes HL, Hoebe K, Hai T, Filippi MD, Karp CL: **ATF3 is a novel regulator of mouse neutrophil migration**. *Blood* 2014, **123**:2084–2093.

290. Watanabe M, Terasawa M, Miyano K, Yanagihara T, Uruno T, Sanematsu F, Nishikimi A, Côté J-F, Sumimoto H, Fukui Y: **DOCK2 and DOCK5 Act Additively in Neutrophils To Regulate Chemotaxis, Superoxide Production, and Extracellular Trap Formation**. *J Immunol* 2014, **193**:5660–5667.

291. Csépányi-Kömi R, Sirokmány G, Geiszt M, Ligeti E: **ARHGAP25, a novel Rac GTPase-activating protein, regulates phagocytosis in human neutrophilic granulocytes**. *Blood* 2012, **119**:573–582.

292. Gloerich M, Bos JL: **Regulating Rap small G-proteins in time and space**. *Trends Cell Biol* 2011, **21**:615–623.

293. Li Y, Yan J, De P, Chang H-C, Yamauchi A, Christopherson KW, Paranavitana NC, Peng X, Kim C, Munugulavadla V, et al.: **Rap1a null mice have altered myeloid cell functions suggesting distinct roles for the closely related Rap1a and 1b proteins**. *J Immunol* 2008, **180**:3612.5-3612.

294. Carbo C, Duerschmied D, Goerge T, Hattori H, Sakai J, Cifuni SM, White GC, Chrzanowska-Wodnicka M, Luo HR, Wagner DD: **Integrin-independent role of CalDAG-GEFI in neutrophil chemotaxis**. *J Leukoc Biol* 2010, **88**:313–319.

295. Stadtmann A, Brinkhaus L, Mueller H, Rossaint J, Bolomini-Vittori M, Bergmeier W, Van Aken H, Wagner DD, Laudanna C, Ley K, et al.: **Rap1a activation by CalDAG-GEFI and p38 MAPK is involved in E-selectin-dependent slow leukocyte rolling**. *Eur J Immunol* 2011, **41**:2074–2085.

296. Kumar S, Xu J, Kumar RS, Lakshmikanthan S, Kapur R, Kofron M, Chrzanowska-Wodnicka M, Filippi MD: **The small GTPase Rap1b negatively regulates neutrophil chemotaxis and transcellular diapedesis by inhibiting Akt activation**. *J Exp Med* 2014, **211**:1741–1758.

297. Kyung SL, So RK, Seoung JP, Kyung HM, Ka YL, Yeong HC, Seung YP, Ok HC, Zhang X, Chang HS, et al.: **Mast cells can mediate vascular permeability through regulation of the PI3K-HIF-1α-VEGF axis**. *Am J Respir Crit Care Med* 2008, **178**:787–797.

298. Di Lorenzo A, Fernández-Hernando C, Cirino G, Sessa WC: **Akt1 is critical for acute inflammation and histamine-mediated vascular leakage**. *Proc Natl Acad Sci U S A* 2009, **106**:14552–14557.

299. Tsuji-Tamura K, Ogawa M: **Inhibition of the PI3K-Akt and mTORC1 signaling pathways promotes the elongation of vascular endothelial cells**. *J Cell Sci* 2016, **129**:1165–1178.

300. Yao H, Duan M, Buch S: **Cocaine-mediated induction of platelet-derived growth factor: Implication for increased vascular permeability**. *Blood* 2011, **117**:2538–2547.

301. Cain RJ, Vanhaesebroeck B, Ridley AJ: **The PI3K p110α isoform regulates endothelial adherens junctions via Pyk2 and Rac1**. *J Cell Biol* 2010, **188**:863–876.

302. Lee KS, Park SJ, Kim SR, Min KH, Jin SM, Puri KD, Lee YC: **Phosphoinositide 3-kinase-δ inhibitor reduces vascular permeability in a murine model of asthma**. *J Allergy Clin Immunol* 2006, **118**:403–409.

303. Cain RJ, Vanhaesebroeck B, Ridley AJ: **Different PI 3-kinase inhibitors have distinct effects on endothelial permeability and leukocyte transmigration**. *Int J Biochem Cell Biol* 2012, **44**:1929–1936.

304. Ong E, Gao XP, Predescu D, Broman M, Malik AB: **Role of phosphatidylinositol 3-kinase-γ in mediating lung neutrophil sequestration and vascular injury induced by E. coli sepsis**. *Am J Physiol - Lung Cell Mol Physiol* 2005, **289**.

305. Frister A, Schmidt C, Schneble N, Brodhun M, Gonnert FA, Bauer M, Hirsch E, Müller JP, Wetzker R, Bauer R: **Phosphoinositide 3-Kinase γ Affects LPS-Induced Disturbance of Blood–Brain Barrier Via Lipid Kinase-Independent Control of cAMP in Microglial Cells**. *NeuroMolecular Med* 2014, **16**:704–713.

306. He HQ, Liao D, Wang ZG, Wang ZL, Zhou HC, Wang MW, Ye RD: **Functional characterization of three mouse formyl peptide receptors**. *Mol Pharmacol* 2013, **83**:389–398.

307. He R, Tan L, Browning DD, Wang JM, Ye RD: **The Synthetic Peptide Trp-Lys-Tyr-Met-Val-d-Met Is a Potent Chemotactic Agonist for Mouse Formyl Peptide Receptor**. *J Immunol* 2000, **165**:4598–4605.

308. Horewicz VV, Crestani S, De Sordi R, Rezende E, Assreuy J: **FPR2/ALX activation reverses LPS-induced vascular hyporeactivity in aorta and increases survival in a pneumosepsis model**. *Eur J Pharmacol* 2015, **746**:267–273.

309. Berkestedt I, Nelson A, Bodelsson M: **Endogenous antimicrobial peptide LL-37 induces human vasodilatation**. In *British Journal of Anaesthesia*. . 2008:803–809.

310. Sasaki T, Irie-Sasaki J, Jones RG, Oliveira-Dos-Santos AJ, Stanford WL, Bolon B, Wakeham A, Itie A, Bouchard D, Kozieradzki I, et al.: **Function of PI3Kγ in thymocyte development, T cell activation, and neutrophil migration**. *Science* 2000, **287**:1040–4046.

311. Okkenhaug K, Bilancio A, Farjot G, Priddle H, Sancho S, Peskett E, Pearce W, Meek SE, Salpekar A, Waterfield MD, et al.: **Impaired B and T cell antigen receptor signaling in p110δ PI 3-kinase mutant mice**. *Science* 2002, **297**:1031–1034.

312. Xu N, Lei X, Liu L: **Tracking neutrophil intraluminal crawling, transendothelial migration and chemotaxis in tissue by intravital video microscopy**. *J Vis Exp* 2011, doi:10.3791/3296.

313. Su Y, Lei X, Wu L, Liu L: **The role of endothelial cell adhesion molecules P-selectin, E-selectin and intercellular adhesion molecule-1 in leucocyte recruitment induced by exogenous methylglyoxal**. *Immunology* 2012, **137**:65–79.

314. Liu L, Cara DC, Kaur J, Raharjo E, Mullaly SC, Jongstra-Bilen J, Jongstra J, Kubes P: **LSP1 is an endothelial gatekeeper of leukocyte transendothelial migration**. *J Exp Med* 2005, **201**:409–418.

315. Lei X, Hossain M, Qadri SM, Liu L: **Different microvascular permeability responses elicited by the CXC chemokines MIP-2 and KC during leukocyte recruitment: Role of LSP1**. *Biochem Biophys Res Commun* 2012, **423**:484–489.

316. Hickey MJ, Issekutz AC, Reinhardt PH, Fedorak RN, Kubes P: **Endogenous interleukin-10 regulates hemodynamic parameters, leukocyte- endothelial cell interactions, and microvascular permeability during endotoxemia**. *Circ Res* 1998, **83**:1124–1131.

317. Bae YS, Ju SA, Kim JY, Seo JK, Baek SH, Kwak JY, Kim BS, Suh PG, Ryu SH: **Trp-Lys-Tyr-Met-Val-D-Met stimulates superoxide generation and killing of Staphylococcus aureus via phospholipase D activation in human monocytes**. *J Leukoc Biol* 1999, **65**:241–248.

318. Zhou H, Andonegui G, Wong CHY, Kubes P: **Role of Endothelial TLR4 for Neutrophil Recruitment into Central Nervous System Microvessels in Systemic Inflammation**. *J Immunol* 2009, **183**:5244–5250.

319. Lieber JG, Webb S, Suratt BT, Young SK, Johnson GL, Keller GM, Worthen GS: **The in vitro production and characterization of neutrophils from embryonic stem cells**. *Blood* 2004, **103**:852–859.

320. Hossain M, Qadri SM, Su Y, Liu L: **ICAM-1-mediated leukocyte adhesion is critical for the activation of endothelial LSP1**. *Am J Physiol - Cell Physiol* 2013, **304**.

321. Chu JY, Dransfield I, Rossi AG, Vermeren S: **Non-canonical PI3K-Cdc42-Pak-Mek-Erk Signaling Promotes Immune-Complex-Induced Apoptosis in Human Neutrophils**. *Cell Rep* 2016, **17**:374–386.

322. Su Y, Qadri SM, Hossain M, Wu L, Liu L: **Uncoupling of eNOS contributes to redox-sensitive leukocyte recruitment and microvascular leakage elicited by methylglyoxal**. *Biochem Pharmacol* 2013, **86**:1762–1774.

323. Hattori H, Subramanian KK, Sakai J, Jia Y, Li Y, Porter TF, Loison F, Sarraj B, Kasorn A, Jo H, et al.: **Small-molecule screen identifies reactive oxygen species as key regulators of neutrophil chemotaxis**. *Proc Natl Acad Sci U S A* 2010, **107**:3546–3551.

324. Lee HY, Kim SD, Shim JW, Kim HJ, Kwon JY, Kim JM, Baek SH, Park JS, Bae YS: **Activation of human monocytes by a formyl peptide receptor 2-derived pepducin**. *FEBS Lett* 2010, **584**:4102–4108.

325. Bo L, Jiang S, Xie Y, Kan H, Song W, Zhao J: **Effect of Vitamin E and omega-3 fatty acids on protecting ambient PM2.5-induced inflammatory response and oxidative stress in vascular endothelial cells**. *PLoS One* 2016, **11**.

326. Zhao Z, Hu J, Gao X, Liang H, Liu Z: **Activation of AMPK attenuates lipopolysaccharide-impaired integrity and function of blood-brain barrier in human brain microvascular endothelial cells**. *Exp Mol Pathol* 2014, **97**:386–392.

327. Abernathy VJ, Webster RO, Dahms TE: **C-reactive protein inhibits increased pulmonary vascular permeability induced by fMLP in isolated rabbit lungs**. *Am J Physiol - Hear Circ Physiol* 1996, **271**.

328. Gao X, Standiford TJ, Rahman A, Newstead M, Holland SM, Dinauer MC, Liu Q, Malik AB: **Role of NADPH Oxidase in the Mechanism of Lung Neutrophil Sequestration and Microvessel Injury Induced by Gram-Negative Sepsis: Studies in $p47^{phox\ -/-}$ and $gp91^{phox\ -/-}$ Mice** . *J Immunol* 2002, **168**:3974–3982.

329. Farivar AS, Krishnadasan B, Naidu B V., Woolley SM, Verrier ED, Mulligan MS: **Alpha chemokines regulate direct lung ischemia-reperfusion injury**. *J Hear Lung Transplant*

2004, **23**:585–591.

330. Kumar P, Shen Q, Pivetti CD, Lee ES, Wu MH, Yuan SY: **Molecular mechanisms of endothelial hyperpermeability: Implications in inflammation**. *Expert Rev Mol Med* 2009, **11**.

331. Rao RM, Yang L, Garcia-Cardena G, Luscinskas FW: **Endothelial-dependent mechanisms of leukocyte recruitment to the vascular wall**. *Circ Res* 2007, **101**:234–247.

332. Zarbock A, Ley K: **Mechanisms and consequences of neutrophil interaction with the endothelium**. *Am J Pathol* 2008, **172**:1–7.

333. Li LF, Lee CS, Liu YY, Chang CH, Lin CW, Chiu LC, Kao KC, Chen NH, Yang CT: **Activation of Src-dependent Smad3 signaling mediates the neutrophilic inflammation and oxidative stress in hyperoxia-augmented ventilator-induced lung injury**. *Respir Res* 2015, **16**.

334. Finsterbusch M, Voisin MB, Beyrau M, Williams TJ, Nourshargh S: **Neutrophils recruited by chemoattractants in vivo induce microvascular plasma protein leakage through secretion of TNF**. *J Exp Med* 2014, **211**:1307–1314.

335. Moreno JJ: **Differential effects of arachidonic and eicosapentaenoic acid-derived eicosanoids on polymorphonuclear transmigration across endothelial cell cultures**. *J Pharmacol Exp Ther* 2009, **331**:1111–1117.

336. Sharp C, Millar AB, Medford ARL: **Advances in understanding of the pathogenesis of acute respiratory distress syndrome**. *Respiration* 2015, **89**:420–434.

337. Martin TR, Pistorese BP, Chi EY, Goodman RB, Matthay MA: **Effects of leukotriene B4 in the human lung. Recruitment of neutrophils into the alveolar spaces without a change in protein permeability**. *J Clin Invest* 1989, **84**:1609–1619.

338. Condliffe AM, Davidson K, Anderson KE, Ellson CD, Crabbe T, Okkenhaug K, Vanhaesebroeck B, Turner M, Webb L, Wymann MP, et al.: **Sequential activation of class IB and class IA PI3K is important for the primed respiratory burst of human but not murine neutrophils**. *Blood* 2005, **106**:1432–1440.

339. Suire S, Condliffe AM, Ferguson GJ, Ellson CD, Guillou H, Davidson K, Welch H, Coadwell J, Turner M, Chilvers ER, et al.: **Gβγs and the Ras binding domain of p110γ are both important regulators of PI3Kγ signalling in neutrophils**. *Nat Cell Biol* 2006, **8**:1303–1309.

340. Deladeriere A, Gambardella L, Pan D, Anderson KE, Hawkins PT, Stephens LR: **The regulatory subunits of PI3Kγ control distinct neutrophil responses**. *Sci Signal* 2015, **8**.

341. Lehmann K, Müller JP, Schlott B, Skroblin P, Barz D, Norgauer J, Wetzker R: **PI3Kγ controls oxidative bursts in neutrophils via interactions with PKCα and p47phox**. *Biochem J* 2009, **419**:603–610.

342. Klabunde RE, Anderson DE: **Role of nitric oxide and reactive oxygen species in platelet-activating factor-induced microvascular leakage**. *J Vasc Res* 2002, **39**:238–245.

343. Li Y, Trush MA: **Diphenyleneiodonium, an NAD(P)H oxidase inhibitor, also potently inhibits mitochondrial reactive oxygen species production**. *Biochem Biophys Res Commun* 1998, **253**:295–299.

344. Batinić-Haberle I, Cuzzocrea S, Rebouças JS, Ferrer-Sueta G, Mazzon E, Di Paola R, Radi R, Spasojević I, Benov L, Salvemini D: **Pure MnTBAP selectively scavenges peroxynitrite over superoxide: Comparison of pure and commercial MnTBAP samples to MnTE-2-PyP in two models of oxidative stress injury, an SOD-specific Escherichia coli model and carrageenan-induced pleurisy**. *Free Radic Biol Med* 2009, **46**:192–201.

345. Zhang H, He J, Kutateladze TG, Sakai T, Sasaki T, Markadieu N, Erneux C, Prestwich GD: **5-Stabilized phosphatidylinositol 3,4,5-trisphosphate analogues bind Grp1 PH, inhibit phosphoinositide phosphatases, and block neutrophil migration**. *ChemBioChem* 2010, **11**:388–395.

346. Mueller H, Stadtmann A, Van Aken H, Hirsch E, Wang D, Ley K, Zarbock A: **Tyrosine kinase Btk regulates E-selectin-mediated integrin activation and neutrophil recruitment by controlling phospholipase C (PLC) γ2 and PI3Kγ pathways**. *Blood* 2010, **115**:3118–3127.

347. Van Keymeulen A, Wong K, Knight ZA, Govaerts C, Hahn KM, Shokat KM, Bourne HR: **To stabilize neutrophil polarity, PIP3 and Cdc42 augment RhoA activity at the back as well as signals at the front**. *J Cell Biol* 2006, **174**:437–445.

348. Anderson KE, Lipp P, Bootman M, Ridley SH, Coadwell J, Rönnstrand L, Lennartsson J, Holmes AB, Painter GF, Thuring J, et al.: **DAPP1 undergoes a PI 3-kinase-dependent cycle of plasma-membrane recruitment and endocytosis upon cell stimulation**. *Curr Biol* 2000, **10**:1403–1412.

349. Zengel P, Nguyen-Hoang A, Schildhammer C, Zantl R, Kahl V, Horn E: **µ-Slide Chemotaxis: A new chamber for long-term chemotaxis studies**. *BMC Cell Biol* 2011, **12**.

350. Kovtun A, Messerer DAC, Scharffetter-Kochanek K, Huber-Lang M, Ignatius A: **Neutrophils in tissue trauma of the skin, bone, and lung: Two sides of the same coin**. *J Immunol Res* 2018, **2018**.

351. Remick DG, Green LB, Newcomb DE, Garg SJ, Bolgos GL, Call DR: **CXC chemokine redundancy ensures local neutrophil recruitment during acute inflammation**. *Am J Pathol* 2001, **159**:1149–1157.

352. Schmidt EP, Lee WL, Zemans RL, Yamashita C, Downey GP: **On, around, and through: Neutrophil-endothelial interactions in innate immunity**. *Physiology* 2011, **26**:334–347.

353. Hayashi F, Means TK, Luster AD: **Toll-like receptors stimulate human neutrophil function**. *Blood* 2003, **102**:2660–2669.

354. Latasiewicz J, Artz A, Jing D, Blanco MP, Currie SM, Avila MV, Schnoor M, Vestweber D: **HS1 deficiency impairs neutrophil recruitment in vivo and activation of the small GTPases Rac1 and Rap1**. *J Leukoc Biol* 2017, **101**:1133–1142.

355. Chen J, Tang H, Hay N, Xu J, Ye RD: **Akt isoforms differentially regulate neutrophil functions**. *Blood* 2010, **115**:4237–4246.

356. Puri KD, Doggett TA, Douangpanya J, Hou Y, Tino WT, Wilson T, Graf T, Clayton E, Turner M, Hayflick JS, et al.: **Mechanisms and implications of phosphoinositide 3-kinase δ in promoting neutrophil trafficking into inflamed tissue**. *Blood* 2004, **103**:3448–3456.

357. Mócsai A, Walzog B, Lowell CA: **Intracellular signalling during neutrophil recruitment**. *Cardiovasc Res* 2015, **107**:373–385.

358. Williams MR, Azcutia V, Newton G, Alcaide P, Luscinskas FW: **Emerging mechanisms of neutrophil recruitment across endothelium**. *Trends Immunol* 2011, **32**:461–469.

359. Rossaint J, Zarbock A: **Tissue-specific neutrophil recruitment into the lung, liver, and kidney**. *J Innate Immun* 2013, **5**:348–357.

360. Kim ND, Luster AD: **The role of tissue resident cells in neutrophil recruitment**. *Trends Immunol* 2015, **36**:547–555.

361. Liu L, Kubes P: **Molecular mechanisms of leukocyte recruitment: Organ-specific mechanisms of action**. *Thromb Haemost* 2003, **89**:213–220.

362. Marki A, Esko JD, Pries AR, Ley K: **Role of the endothelial surface layer in neutrophil recruitment**. *J Leukoc Biol* 2015, **98**:503–515.

363. Hossain M, Qadri SM, Xu N, Su Y, Cayabyab FS, Heit B, Liu L: **Endothelial LSP1 Modulates Extravascular Neutrophil Chemotaxis by Regulating Nonhematopoietic Vascular PECAM-1 Expression**. *J Immunol* 2015, **195**:2408–2416.

364. Zen K, Parkos CA: **Neutrophil migration across endothelium: Transcellular or paracellular?** *Blood* 2005, **106**:394–395.

365. Vestweber D, Winderlich M, Cagna G, Nottebaum AF: **Cell adhesion dynamics at endothelial junctions: VE-cadherin as a major player**. *Trends Cell Biol* 2009, **19**:8–15.

366. Barreiro O, Zamai M, Yáñez-Mó M, Tejera E, López-Romero P, Monk PN, Gratton E, Caiolfa VR, Sánchez-Madrid F: **Endothelial adhesion receptors are recruited to adherent leukocytes by inclusion in preformed tetraspanin nanoplatforms**. *J Cell Biol* 2008, **183**:527–542.

367. Carman C V., Springer TA: **A transmigratory cup in leukocyte diapedesis both through individual vascular endothelial cells and between them**. *J Cell Biol* 2004, **167**:377–388.

368. Palo J, Rauvala H, Finne J, Haltia M, Palmgren K: **Hyperexcretion of free N-acetylneuraminic acid - a novel type of sialuria**. *Clin Chim Acta* 1985, **145**:237–242.

369. Cho H, Thorvaldsen JL, Chu Q, Feng F, Birnbaum MJ: **Akt1/PKBα Is Required for Normal Growth but Dispensable for Maintenance of Glucose Homeostasis in Mice**. *J Biol Chem* 2001, **276**:38349–38352.

370. Yang ZZ, Tschopp O, Hemmings-Mieszczak M, Feng J, Brodbeck D, Perentes E, Hemmings BA: **Protein kinase Bα/Akt1 regulates placental development and fetal growth**. *J Biol Chem* 2003, **278**:32124–32131.

371. Chen WS, Xu PZ, Gottlob K, Chen ML, Sokol K, Shiyanova T, Roninson I, Weng W, Suzuki R, Tobe K, et al.: **Growth retardation and increased apoptosis in mice with homozygous disruption of the akt1 gene**. *Genes Dev* 2001, **15**:2203–2208.

372. Pierau M, Na SY, Simma N, Lowinus T, Marx A, Schraven B, Bommhardt UH: **Constitutive Akt1 signals attenuate B-cell receptor signaling and proliferation, but enhance B-cell migration and effector function**. *Eur J Immunol* 2012, **42**:3381–3393.

373. Koh ALY, Sun CX, Zhu F, Glogauer M: **The role of Rac1 and Rac2 in bacterial killing**. *Cell Immunol* 2005, **235**:92–97.

374. Gu Y, Filippi MD, Cancelas JA, Siefring JE, Williams EP, Jasti AC, Harris CE, Lee AW, Prabhakar R, Atkinson SJ, et al.: **Hematopoietic Cell Regulation by Rac1 and Rac2 Guanosine Triphosphatases**. *Science* 2003, **302**:445–449.

375. Filippi MD, Szczur K, Harris CE, Berclaz PY: **Rho GTPase Rac1 is critical for neutrophil migration into the lung**. *Blood* 2007, **109**:1257–1264.

376. Marshall AJ, Zhang T, Al-Alwan M: **Regulation of B-lymphocyte activation by the PH domain adaptor protein Bam32/DAPP1**. *Biochem Soc Trans* 2007, **35**:181–182.

377. Niiro H, Clark EA: **Branches of the B cell antigen receptor pathway are directed by protein conduits Bam32 and Carma1**. *Immunity* 2003, **19**:637–640.

378. Krahn AK, Ma K, Hou S, Duronio V, Marshall AJ: **Two Distinct Waves of Membrane-Proximal B Cell Antigen Receptor Signaling Differentially Regulated by Src Homology 2-Containing Inositol Polyphosphate 5-Phosphatase**. *J Immunol* 2004, **172**:331–339.

379. Winther M, Dahlgren C, Forsman H: **Formyl Peptide Receptors in Mice and Men:**

Similarities and Differences in Recognition of Conventional Ligands and Modulating Lipopeptides. *Basic Clin Pharmacol Toxicol* 2018, **122**:191–198.

380. Bae YS, Kim Y, Kim Y, Kim JH, Suh PG, Ryu SH: **Trp-Lys-Tyr-Met-Val-D-Met is a chemoattractant for human phagocytic cells**. *J Leukoc Biol* 1999, **66**:915–922.
381. Germann UA, Furey BF, Markland W, Hoover RR, Aronov AM, Roix JJ, Hale M, Boucher DM, Sorrell DA, Martinez-Botella G, et al.: **Targeting the MAPK signaling pathway in cancer: Promising preclinical activity with the novel selective ERK1/2 inhibitor BVD-523 (ulixertinib)**. *Mol Cancer Ther* 2017, **16**:2351–2363.
382. Huizinga T, Nigrovic P, Ruderman E, Schulze-Koops H: **Mitochondrial reactive oxygen species promote production of proinflammatory cytokines and are elevated in TNFR1-associated periodic syndrome (TRAPS): Commentary**. *Int J Adv Rheumatol* 2011, **9**:108.
383. Kuwabara WMT, Zhang L, Schuiki I, Curi R, Volchuk A, Alba-Loureiro TC: **NADPH oxidase-dependent production of reactive oxygen species induces endoplasmatic reticulum stress in neutrophil-like HL60 cells**. *PLoS One* 2015, **10**.
384. Hara K, Holland S, Woo J: **Effects of Exogenous Reactive Oxygen Species Scavengers on the Survival of Escherichia coli B23 during Exposure to UV-A radiation**. *J Exp Microbiol Immunol (JEMI)* 2004, **12**:62–66.
385. McLeish KR, Uriarte SM, Tandon S, Creed TM, Le J, Ward RA: **Exocytosis of neutrophil granule subsets and activation of prolyl isomerase 1 are required for respiratory burst priming**. *J Innate Immun* 2013, **5**:277–289.
386. Miralda I, Uriarte SM, McLeish KR: **Multiple phenotypic changes define neutrophil priming**. *Front Cell Infect Microbiol* 2017, **7**.
387. Yao Y, Matsushima H, Ohtola JA, Geng S, Lu R, Takashima A: **Neutrophil Priming Occurs in a Sequential Manner and Can Be Visualized in Living Animals by Monitoring IL-1β Promoter Activation**. *J Immunol* 2015, **194**:1211–1224.
388. Uriarte SM, Rane MJ, Luerman GC, Barati MT, Ward RA, Nauseef WM, McLeish KR: **Granule Exocytosis Contributes to Priming and Activation of the Human Neutrophil**

Respiratory Burst. *J Immunol* 2011, **187**:391–400.

389. Rane MJ, Carrithers SL, Arthur JM, Klein JB, McLeish KR: **Formyl Peptide Receptors Are Coupled to Multiple Mitogen-Activated Protein Kinase Cascades by Distinct Signal Transduction Pathways: Role in Activation of Reduced Nicotinamide Adenine Dinucleotide Oxidase**. *J Immunol* 1997, **159**:5070–5078.

390. Monaghan-Benson E, Burridge K: **The regulation of vascular endothelial growth factor-induced microvascular permeability requires Rac and reactive oxygen species**. *J Biol Chem* 2009, **284**:25602–25611.

391. Moldovan L, Moldovan NI, Sohn RH, Parikh SA, Goldschmidt-Clermont PJ: **Redox changes of cultured endothelial cells and actin dynamics**. *Circ Res* 2000, **86**:549–557.

392. Nguyen GT, Green ER, Mecsas J: **Neutrophils to the ROScue: Mechanisms of NADPH oxidase activation and bacterial resistance**. *Front Cell Infect Microbiol* 2017, **7**.

393. El Benna J, Faust LP, Babior BM: **The phosphorylation of the respiratory burst oxidase component p47(phox) during neutrophil activation. Phosphorylation of sites recognized by protein kinase C and by proline-directed kinases**. *J Biol Chem* 1994, **269**:23431–23436.

394. Son Y, Cheong Y-K, Kim N-H, Chung H-T, Kang DG, Pae H-O: **Mitogen-Activated Protein Kinases and Reactive Oxygen Species: How Can ROS Activate MAPK Pathways?** *J Signal Transduct* 2011, **2011**:1–6.

395. Wrzaczek M, Brosché M, Kangasjärvi J: **ROS signaling loops - production, perception, regulation**. *Curr Opin Plant Biol* 2013, **16**:575–582.

396. Vandercappellen J, Van Damme J, Struyf S: **The role of CXC chemokines and their receptors in cancer**. *Cancer Lett* 2008, **267**:226–244.

397. Wong M, Ziring D, Korin Y, Desai S, Kim S, Lin J, Gjertson D, Braun J, Reed E, Singh RR: **TNFα blockade in human diseases: Mechanisms and future directions**. *Clin Immunol* 2008, **126**:121–136.

398. Kakutani R, Adachi Y, Takata H, Kuriki T, Ohno N: **Essential role of Toll-like receptor 2**

in macrophage activation by glycogen. *Glycobiology* 2012, **22**:146–159.

399. Pinho V, de Castro Russo R, Amaral FA, de Sousa LP, Barsante MM, de Souza DG, Alves-Filho JC, Cara DC, Hayflick JS, Rommel C, et al.: **Tissue- and stimulus-dependent role of phosphatidylinositol 3-kinase isoforms for neutrophil recruitment induced by chemoattractants in vivo**. *J Immunol* 2008, **180**:7775.1-7775.
400. Ferrandi C, Ardissone V, Ferro P, Rückle T, Zaratin P, Ammannati E, Hauben E, Rommel C, Cirillo R: **Phosphoinositide 3-kinase γ inhibition plays a crucial role in early steps of inflammation by blocking neutrophil recruitment**. *J Pharmacol Exp Ther* 2007, **322**:923–930.
401. Carlos TM, Harlan JM: **Leukocyte-endothelial adhesion molecules**. *Blood* 1994, **84**:2068–2101.
402. Enoksson M, Lyberg K, Möller-Westerberg C, Fallon PG, Nilsson G, Lunderius-Andersson C: **Mast Cells as Sensors of Cell Injury through IL-33 Recognition**. *J Immunol* 2011, **186**:2523–2528.
403. Supajatura V, Ushio H, Nakao A, Akira S, Okumura K, Ra C, Ogawa H: **Differential responses of mast cell Toll-like receptors 2 and 4 in allergy and innate immunity**. *J Clin Invest* 2002, **109**:1351–1359.
404. Cheng X, Veverka V, Radhakrishnan A, Waters LC, Muskett FW, Morgan SH, Huo J, Yu C, Evans EJ, Leslie AJ, et al.: **Structure and interactions of the human programmed cell death 1 receptor**. *J Biol Chem* 2013, **288**:11771–11785.
405. Nussbaum C, Bannenberg S, Keul P, Gräler MH, Gonçalves-De-Albuquerque CF, Korhonen H, Von Wnuck Lipinski K, Heusch G, De Castro Faria Neto HC, Rohwedder I, et al.: **Sphingosine-1-phosphate receptor 3 promotes leukocyte rolling by mobilizing endothelial P-selectin**. *Nat Commun* 2015, **6**.
406. Andriopoulou P, Navarro P, Zanetti A, Lampugnani MG, Dejana E: **Histamine induces tyrosine phosphorylation of endothelial cell-to-cell adherens junctions**. *Arterioscler Thromb Vasc Biol* 1999, **19**:2286–2297.

407. Abtin A, Jain R, Mitchell AJ, Roediger B, Brzoska AJ, Tikoo S, Cheng Q, Ng LG, Cavanagh LL, Von Andrian UH, et al.: **Perivascular macrophages mediate neutrophil recruitment during bacterial skin infection**. *Nat Immunol* 2014, **15**:45–53.

408. Stark K, Eckart A, Haidari S, Tirniceriu A, Lorenz M, Von Brühl ML, Gärtner F, Khandoga AG, Legate KR, Pless R, et al.: **Capillary and arteriolar pericytes attract innate leukocytes exiting through venules and "instruct" them with pattern-recognition and motility programs**. *Nat Immunol* 2013, **14**:41–51.

409. Proebstl D, Voisin MB, Woodfin A, Whiteford J, D'Acquisto F, Jones GE, Rowe D, Nourshargh S: **Pericytes support neutrophil subendothelial cell crawling and breaching of venular walls in vivo**. *J Exp Med* 2012, **209**:1219–1234.

410. Lämmermann T, Afonso P V., Angermann BR, Wang JM, Kastenmüller W, Parent CA, Germain RN: **Neutrophil swarms require LTB4 and integrins at sites of cell death in vivo**. *Nature* 2013, **498**:371–375.

411. Puls A, Eliopoulos AG, Nobes CD, Bridges T, Young LS, Hall A: **Activation of the small GTPase Cdc42 by the inflammatory cytokines TNFα and IL-1, and by the Epstein-Barr virus transforming protein LMP1**. *J Cell Sci* 1999, **112**:2983–2992.

412. Slaba I, Wang J, Kolaczkowska E, Mcdonald B, Lee WY, Kubes P: **Imaging the dynamic platelet-neutrophil response in sterile liver injury and repair in mice**. *Hepatology* 2015, **62**:1593–1605.

413. Weber C, Springer TA: **Neutrophil accumulation on activated, surface-adherent platelets in flow is mediated by interaction of Mac-1 with fibrinogen bound to αIIbβ3 and stimulated by platelet-activating factor**. *J Clin Invest* 1997, **100**:2085–2093.

414. Bennett JS: **Structure and function of the platelet integrin αIIbβ 3**. *J Clin Invest* 2005, **115**:3363–3369.

415. Ruggeri ZM, Mendolicchio GL: **Adhesion mechanisms in platelet function**. *Circ Res* 2007, **100**:1673–1685.

416. Vanichakarn P, Blair P, Wu C, Freedman JE, Chakrabarti S: **Neutrophil CD40 enhances**

platelet-mediated inflammation. *Thromb Res* 2008, **122**:346–358.

417. Von Hundelshausen P, Weber C: **Platelets as immune cells: Bridging inflammation and cardiovascular disease**. *Circ Res* 2007, **100**:27–40.

418. Hartwig H, Drechsler M, Lievens D, Kramp B, von Hundelshausen P, Lutgens E, Weber C, Döring Y, Soehnlein O: **Platelet-derived PF4 reduces neutrophil apoptosis following arterial occlusion**. *Thromb Haemost* 2013, **111**:562–564.